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(54) Title: A LIPASE EXPRESSED IN ENDOTHELIAL CELLS AND METHODS FOR ITS USE

(57) Abstract

The present invention relates to a novel lipase expressed in endothelial cells. In particular, the invention relates to polynucleotides that encode the polypeptides, the polypeptides, antibodies directed to the polypeptides, and methods of diagnosis and treatment of vascular disorders, lipidemia, diabetes and associated complications, obesity, restenosis and cancer based on gene expression and function.

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A LIPASE EXPRESSED IN ENDOTHELIAL CELLS AND METHODS FOR ITS USE

1. INTRODUCTION

The present invention relates to a novel lipase expressed in endothelial cells, which is hereinafter referred to as endothelial cell lipase (EL). In particular, the invention relates to polynucleotides that encode the EL polypeptides, the EL polypeptides, antibodies directed to the EL polypeptides, and methods of diagnosis and treatment of vascular disorders, lipidemia, diabetes and associated complications, obesity, restenosis and cancer based on the *EL* gene expression and function.

2. BACKGROUND OF THE INVENTION

2.1. ENDOTHELIAL CELL BIOLOGY AND BLOOD VESSEL DEVELOPMENT

The endothelium occupies a pivotal position at the interface between the circulating humoral and cellular elements of the blood, and the solid tissues which constitute the various organs. In this unique position, endothelial cells regulate a large number of critical processes. Such processes include leukocyte adherence and transit through the blood vessel wall, local control of blood vessel tone, modulation of the immune response, the balance between thrombosis and thrombolysis, and new blood vessel development (Bevilacqua et al., 1993, *J. Clin. Invest* 91:379-387; Folkman et al., 1987, *Science* 235:442-447; Folkman et al., 1992, J. Biol. Chem. 267:10931-10934; Gimbrone, 1986, Churchill Livingstone, London; Issekutz, 1992, *Curr. Opin. Immunol.* 4:287-293; Janssens et al., 1992, *J. Biol. Chem.* 267:14519-14522; Lamas et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:6348-6352; Luscher et al., 1992, *Hypertension* 19:117-130; Williams et al., 1992, *Am. Rev. Respir. Dis.* 146:S45-S50; Yanagisawa, et al., 1988, *Nature* 332:411-415).

Endothelial cell dysfunction has been postulated as a central feature of vascular diseases such as hypertension and atherosclerosis. In this context, the ability of the endothelium to synthesize smooth muscle cell mitogens and factors which control smooth muscle contraction has received much attention (Janssens et al., 1992, *J. Biol. Chem.* 267:14519-14522; Lamas et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:6348-6352; Luscher

et al., 1992, *Hypertension* 19:117-130; Raines et al., 1993, *Br. Heart J.* 69:S30-S37; Yanagisawa et al., 1988, *Nature* 332:411-415). The endothelial cell has also become the focus of attention in the study of diseases which are not primarily vascular in nature. Diverse disease processes such as adult respiratory distress syndrome, septic shock, solid tumor formation, tumor cell metastasis, rheumatoid arthritis, and transplant rejection are now understood to be related to normal or aberrant function of the endothelial cell. A rapidly increasing number of pharmacologic agents are being developed whose primary therapeutic action will be to alter endothelial cell function. In addition, recent attention on gene therapy has focused on the endothelial cell (Nabel et al., 1991, *J. Am. Coll. Cardiol.* 17:189B-194B). Transfer of genes into the endothelial cell may afford a therapeutic strategy for vascular disease, or the endothelium may serve simply as a convenient cellular factory for a missing blood borne factor. Hence, information regarding fundamental processes in the endothelial cell will aid the understanding of disease processes and allow more effective therapeutic strategies.

All blood vessels begin their existence as a capillary, composed of only endothelial cells. Much of the molecular research investigating the role of endothelial cells in blood vessel development has focused on this process in the adult organism, in association with pathological conditions. In these situations, new blood vessels are formed by budding and branching of existing vessels. This process, which depends on endothelial cell division, has been termed angiogenesis. Research on this process has focused primarily on small proteins which are growth factors for endothelial cells (Folkman et al., 1987, *Science* 235:442-447; Folkman et al., 1992, *J. Biol. Chem.* 267:10931-10934).

2.2. LIPOPROTEIN LIPASES

Triglyceride lipases are lipolytic enzymes that hydrolyze the ester bond of triglycerides. Lipases have been found in diverse species, including animals, plants and prokaryotes. In mammals, lipases have been divided into three tissue-specific classes: pancreatic, hepatic and lipoprotein lipase (LPL) found primarily in fat and muscle. All lipases contain a highly conserved serine active site (Blow, 1990, Nature 343:694; Persson *et al.*, 1989, Eur. J. Biochem. 179:39).

Lipoproteins are spherical particles composed of lipid and protein molecules (Ginsberg, 1994, Lipid Disorders 78:1). The major types of lipid in lipoproteins are cholesterol, triglycerides and phospholipids. Apoproteins or apolipoproteins occupy the surface of lipoproteins, and as such, they play important roles in lipoprotein metabolism. Lipoproteins have been divided into several classes based on their physical and chemical characteristics: chylomicrons, very low density lipoproteins (VLDL), intermediate-density lipoproteins (IDS), low density lipoproteins (LDL) and high density lipoproteins (HDL).

LPL hydrolyzes triglycerides in circulating lipoproteins to produce mono- and diglycerides, free fatty acids, and chylomicron remnants (Santamarina-Fojo, 1994, Current Opin. in Lipid. 5:117). LPL are produced in adipose tissue, muscle, mammary gland, brain and macrophages (Yla-Herttuala *et al.*, 1991, Proc. Natl. Acad. Sci. U.S.A. 88:10143), and are secreted by the cells for attachment to endothelial cells in the lumen of capillaries and arteries by binding to heparin sulfate proteoglycans. Prior to the present invention, endothelial cells were not known to synthesize LPL.

Patients with LPL deficiency such as familial chylomicronemia syndrome are characterized with hypertriglyceridemia and pancreatitis (Santamarina-Fojo and Dugi, 1994, Current Opin. in Lipid. 5:117). This condition results from mutations in LPL genes. In animals, marked hypertriglyceridemia has been shown to cause fatality. Furthermore, LPL defects have been linked to diabetes and renal failure. In a rat model, induction of LPL activity protected against coronary artery lesions (Tsutsumi *et al.*, 1993, J. Clin. Invest. 92:411). A mutation in a human LPL gene has been associated with reduced LPL catalytic activities and enhanced susceptibility to atherosclerosis (Reymer *et al.*, 1995, Nature Genetics 10:28).

On the contrary, LPL has been proposed as capable of promoting atherogenesis by producing remnant lipoproteins in the artery wall (Yla-Herttuala *et al.*, 1991, Proc. Natl. Acad. Sci. U.S.A. 88:10143; Zilversmit, 1979, Circulation 60:473). Therefore, whether LPL is beneficial or detrimental to an individual may depend on a large number of factors, including the level of LPL expression, the location of LPL activities and its interactions with specific substrates (Goldberg, 1996, J. Lipid Res. 37:693).

3. SUMMARY OF THE INVENTION

The present invention relates to a novel lipase expressed in endothelial cells known as EL. In particular, it relates to polynucleotides encoding EL polypeptides, EL polypeptides, antibodies to EL polypeptides, methods of detecting EL as an endothelial cell marker, and methods of diagnosing vascular disorders based on expression levels or mutation of *EL*, as well as methods of treating vascular disorders, atherosclerosis and cancer.

The invention is based, in part, on Applicants' discovery that *EL* gene expression is upregulated in endothelial cells undergoing differentiation such as angiogenesis. While EL shares sequence homology with LPL and HL, it is unique in its primary sequence. Its expression is detected in both human and mouse tissues, including the placenta, liver, kidney, lung and testis. EL possesses phospholipase A1 activities, but does not exhibit triglyceride lipase activities.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-1C: The nucleotide sequence (SEQ ID NO:1) of a human *EL* cDNA clone and the deduced amino acid sequence (SEQ ID NO:2) corresponding to the open reading frames. The initiating methionine is located at position #1 of the amino acid sequence (SEQ ID NO:2). The signal peptide is predicted to contain a cleavage site between residue #18 and #19. The translation stop codon is indicated by the asterisk at position #501.

Figure 2: Amino acid sequence alignment of human EL (SEQ ID NO:2) with murine EL (SEQ ID NO:3) and a human LPL (SEQ ID NO:4). The predicted signal peptide cleavage site of EL is indicated by the dashed downward arrow at amino acid residue #19, whereas the signal sequence cleavage site of human LPL is indicated by an upward dashed arrow. Conserved cysteines are indicated by dots, and the catalytic triad is indicated by asterisks.

Figure 3A-3B: Northern blot analysis of *EL* in adult tissues. The blot was probed with a radiolabeled human or mouse *EL* cDNA fragment. A predominant band is observed at about 4.4 kilobases in both human and mouse samples. Figure 3A: human tissues. Figure 3B: mouse tissues.

Figure 4A-4B: Northern blot analysis of *EL* in embryonic tissues using the same radiolabeled probes as in Figure 3A and 3B. Figure 4A: human tissues. Figure 4B: mouse tissues.

5. DETAILED DESCRIPTION OF THE INVENTION

For clarity of discussion, the invention is described in the subsections below by way of examples for human and mouse *EL* genes and their encoded products. However, the findings disclosed herein may be analogously applied to other homologous members of the EL family in all species.

5.1. THE *EL* CODING SEQUENCE

The present invention relates to nucleic acid molecules that encode polypeptides referred to as EL. In a specific embodiment by way of example in Section 6, infra, full length human and mouse *EL* nucleic acid molecules were cloned, and their nucleotide and deduced amino acid sequences characterized (SEQ ID NOS:1-3 and 5). While *EL* shares sequence homology with hepatic lipase and LPL from different species, the nucleotide coding sequence and deduced amino acid sequence of EL are structurally unique. Both mature human and mouse EL proteins contain 482 amino acids. In accordance with the invention, any nucleotide sequence which encodes the amino acid sequence of the human or mouse EL gene product can be used to generate recombinant molecules which direct the expression of the *EL* genes. Additionally, the invention also relates to a fusion polynucleotide between an *EL* coding sequence and a second coding sequence for a heterologous protein.

In order to clone full length homologous cDNA sequences from any species encoding the entire EL cDNA or to clone family members or variant forms such as allelic variants, labeled DNA probes made from fragments corresponding to any part of the cDNA

sequences disclosed herein may be used to screen a cDNA library derived from endothelial cells, liver or placenta. More specifically, oligonucleotides corresponding to either the 5' or 3' terminus of the coding sequence may be used to obtain longer nucleotide sequences. Briefly, the library may be plated out to yield a maximum of 30,000 pfu for each 150 mm plate. Approximately 40 plates may be screened. The plates are incubated at 37°C until the plaques reach a diameter of 0.25 mm or are just beginning to make contact with one another (3-8 hours). Nylon filters are placed onto the soft top agarose and after 60 seconds, the filters are peeled off and floated on a DNA denaturing solution consisting of 0.4N sodium hydroxide. The filters are then immersed in neutralizing solution consisting of 1M Tris HCℓ, pH 7.5, before being allowed to air dry. The filters are prehybridized in casein hybridization buffer containing 10% dextran sulfate, 0.5M NaCl, 50mM Tris HCL, pH 7.5, 0.1% sodium pyrophosphate, 1% casein, 1% SDS, and denatured salmon sperm DNA at 0.5 mg/ml for 6 hours at 60°C. The radiolabelled probe is then denatured by heating to 95°C for 2 minutes and then added to the prehybridization solution containing the filters. The filters are hybridized at 60°C for 16 hours. The filters are then washed in 1X wash mix (10X wash mix contains 3M NaCl, 0.6M Tris base, and 0.02M EDTA) twice for 5 minutes each at room temperature, then in 1X wash mix containing 1% SDS at 60°C for 30 minutes, and finally in 0.3X wash mix containing 0.1% SDS at 60°C for 30 minutes. The filters are then air dried and exposed to x-ray film for autoradiography. After developing, the film is aligned with the filters to select a positive plaque. If a single, isolated positive plaque cannot be obtained, the agar plug containing the plaques will be removed and placed in lambda dilution buffer containing 0.1M NaCl, 0.01M magnesium sulfate, 0.035M Tris HCl, pH 7.5, 0.01% gelatin. The phage may then be replated and rescreened to obtain single, well isolated positive plaques. Positive plaques may be isolated and the cDNA clones sequenced using primers based on the known cDNA sequence. This step may be repeated until a full length cDNA is obtained.

It may be necessary to screen multiple cDNA libraries from different tissues to obtain a full length cDNA. In the event that it is difficult to identify cDNA clones encoding the complete 5' terminal coding region, an often encountered situation in cDNA cloning, the RACE (Rapid Amplification of cDNA Ends) technique may be used. RACE is a proven

PCR-based strategy for amplifying the 5' end of incomplete cDNAs. 5'-RACE-Ready RNA synthesized from human placenta containing a unique anchor sequence is commercially available (Clontech). To obtain the 5' end of the cDNA, PCR is carried out on 5'-RACE-Ready cDNA using the provided anchor primer and the 3' primer. A secondary PCR is then carried out using the anchored primer and a nested 3' primer according to the manufacturer's instructions. Once obtained, the full length cDNA sequence may be translated into amino acid sequence and examined for certain landmarks such as a continuous open reading frame flanked by translation initiation and termination sites, a serine active site, a potential signal sequence and a heparin-binding site, and finally overall structural similarity to the *EL* genes disclosed herein.

Alternatively, a labeled probe may be used to screen a genomic library derived from any organism of interest using appropriate stringent conditions as described, *infra*.

Isolation of an *EL* coding sequence or a homologous sequence may be carried out by the polymerase chain reactions (PCR) using two degenerate oligonucleotide primer pools designed on the basis of the *EL* coding sequences disclosed herein. The template for the reaction may be cDNA obtained by reverse transcription (RT) of mRNA prepared from, for example, human or non-human cell lines or tissues known or suspected to express an *EL* gene allele.

The PCR product may be subcloned and sequenced to ensure that the amplified sequences represent the sequences of an *EL* coding sequence. The PCR fragment may then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to screen a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to isolate genomic clones via the screening of a genomic library.

PCR technology may also be utilized to isolate full length cDNA sequences. For example, RNA may be isolated, following standard procedures, from an appropriate cellular or tissue source. A RT reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" with guanines using a standard terminal transferase reaction, the hybrid may be digested with RNAase H, and

second strand synthesis may then be primed with a poly-C primer. Thus, cDNA sequences upstream of the amplified fragment may easily be isolated.

A cDNA clone of a mutant or allelic variant of the *EL* gene may be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying the mutant *EL* allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant *EL* allele to that of the normal *EL* allele, the mutation(s) responsible for the loss or alteration of function of the mutant *EL* gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry a mutant *EL* allele, or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant *EL* allele. An unimpaired *EL* gene or any suitable fragment thereof may then be labeled and used as a probe to identify the corresponding mutant *EL* allele in such libraries. Clones containing the mutant *EL* gene sequences may then be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant *EL* allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against the normal *EL* gene product, as described, below, in Section 5.4. (For screening techniques, see, for example, Harlow and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.)

In cases where an *EL* mutation results in an expressed gene product with altered function (*e.g.*, as a result of a missense), a polyclonal set of anti-*EL* gene product antibodies

are likely to cross-react with the mutant *EL* gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

The invention also relates to isolated or purified polynucleotides having at least 12 nucleotides (*i.e.*, a hybridizable portion) of an *EL* coding sequence or its complement. In other embodiments, the polynucleotides contain at least 25 (continuous) nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, or 200 nucleotides of an *EL* coding sequence, or a full-length *EL* coding sequence. Nucleic acids can be single or double stranded. Additionally, the invention relates to polynucleotides that selectively hybridize to a complement of the foregoing coding sequences. In preferred embodiments, the polynucleotides contain at least 12, 25, 50, 100, 150 or 200 nucleotides or the entire length of an *EL* coding sequence.

In a specific embodiment, a polynucleotide which hybridizes to an *EL* coding sequence (*e.g.*, having sequence SEQ ID NO:1 or 5) or its complement under conditions of low stringency is provided. By way of example and not limitation, exemplary conditions of low stringency are as follows (Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

In another specific embodiment, a polynucleotide which hybridizes to an *EL* coding sequence or its complement under conditions of high stringency is provided. By way of

example and not limitation, exemplary conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency which may be used are well known in the art.

In another specific embodiment, a polynucleotide which hybridizes to an *EL* coding sequence or its complement under conditions of moderate stringency is provided. Exemplary conditions of moderate stringency are as follows: Filters containing DNA are pretreated for 6 h at 55°C in a solution containing 6X SSC, 5X Denhart's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 55°C, and then washed twice for 30 minutes at 60°C in a solution containing 1X SSC and 0.1% SDS. Filters are blotted dry and exposed for autoradiography. Other conditions of moderate stringency which may be used are well-known in the art.

5.2. PRODUCTS ENCODED BY *EL* CODING SEQUENCES

In accordance with the invention, an *EL* polynucleotide which encodes full length EL polypeptides, mutant polypeptides, peptide fragments of EL, EL fusion proteins or functional equivalents thereof, may be used to generate recombinant DNA molecules that direct the expression of EL polypeptides, mutant polypeptides, EL peptide fragments, EL fusion proteins or a functional equivalent thereof, in appropriate host cells. Such polynucleotides, as well as other polynucleotides which selectively hybridize to at least a part of such *EL* polynucleotides or their complements, may also be used to produce EL polypeptides or in nucleic acid hybridization assays, such as Southern and Northern blot

analyses, etc. The polypeptide products encoded by such polynucleotides may be naturally occurring or altered by molecular manipulation of the coding sequence.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent EL amino acid sequence (SEQ ID NO:2 or 3), may be used in the practice of the invention for the cloning and expression of EL proteins. Such DNA sequences include those which are capable of hybridizing to the human or mouse *EL* coding sequence or its complementary sequence under low, moderate or high stringency conditions as described in Section 5.1, *supra*.

Altered nucleotide sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may contain deletions, additions or substitutions of amino acid residues within its sequence, which result in a silent change thus producing a functionally equivalent EL protein. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine, histidine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the following: glycine, asparagine, glutamine, serine, threonine and tyrosine; and amino acids with nonpolar head groups include alanine, valine, isoleucine, leucine, phenylalanine, proline, methionine and tryptophan.

The nucleotide sequences of the invention may be engineered in order to alter an *EL* coding sequence for a variety of ends, including but not limited to, alterations which modify processing and expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, *e.g.*, site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation, etc. Alterations may also affect one or more biologic activities of EL. For example, cysteine residues can be deleted or substituted with another amino acid to eliminate disulfide bridges.

Based on the domain organization of the EL protein, a large number of EL mutant polypeptides can be constructed by rearranging the nucleotide sequences that encode the EL

domains. Since the serine-active site of EL is known to be involved in substrate binding, and a heparin binding site interacts with proteoglycans, EL mutant polypeptides containing one of these regions can be generated and their functional activities compared.

In another embodiment of the invention, an *EL* coding sequence, a modified *EL* sequence or a truncated *EL* coding sequence corresponding to a specific domain may be ligated to a heterologous sequence to produce a fusion protein. For example, for screening of peptide libraries for molecules that bind EL, it may be useful to encode a chimeric EL protein expressing a heterologous epitope that is recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between an EL sequence and the heterologous protein sequence, so that the EL may be cleaved and separated from the heterologous moiety. A heterologous moiety includes, but is not limited to, immunoglobulin constant domain which prolongs *in vivo* half life of the fusion protein, a cell surface molecule which anchors the fusion protein to the cell membrane, and a detectable label such as a fluorescent protein or an enzyme.

In a specific embodiment of the invention, the coding sequence of *EL* could be synthesized in whole or in part, using chemical methods well known in the art. See, for example, Caruthers *et al.*, 1980, *Nuc. Acids Res. Symp. Ser.* 7:215-233; Crea and Horn, 180, *Nuc. Acids Res. 9(10)*:2331; Matteucci and Caruthers, 1980, *Tetrahedron Letter 21*:719; and Chow and Kempe, 1981, *Nuc. Acids Res. 9(12)*:2807-2817. Alternatively, the polypeptide itself could be produced using chemical methods to synthesize an EL amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. (*e.g.*, see Creighton, 1983, *Proteins Structures And Molecular Principles*, W.H. Freeman and Co., N.Y. pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; see Creighton, 1983, *Proteins, Structures and Molecular Principles*, W.H. Freeman and Co., N.Y., pp. 34-49).

In a specific embodiment of the invention, a polypeptide containing at least 10 (continuous) amino acids of the EL protein is provided. In other embodiments, the polypeptide may contain at least 20 or 50 amino acids. In specific embodiments, such

polypeptides do not contain more than 100, 150 or 200 amino acids. Derivatives or analogs of the polypeptides include, but are not limited to, molecules containing regions that are substantially homologous to the EL protein or fragments thereof (*e.g.*, in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or product encoded by a polynucleotide that is capable of hybridizing to a naturally-occurring coding sequence, under highly stringent, moderately stringent, or low stringent conditions.

The derivatives and analogs of EL protein can be produced by various methods known in the art. The manipulations which result in their production can occur at the nucleic acid or protein level. For example, a cloned coding sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of a polynucleotide encoding a derivative or analog, care should be taken to ensure that the modified coding sequence remains within the same translational reading frame as the antigen, uninterrupted by translational stop signals, in the coding region where the functional domain is encoded.

Additionally, the coding sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), and the like.

Manipulations may also be made at the protein level. Included within the scope of the invention are protein fragments or other derivatives or analogs which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage,

linkage to a heterologous polypeptide or another antigen. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to, specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives can be chemically synthesized. Nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the sequence. Non-classical amino acids include, but are not limited to, the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ϵ-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In a specific embodiment, the derivative is a chimeric or fusion protein containing EL or a fragment thereof joined at its amino- or carboxy-terminus to a heterologous protein via a peptide bond. Alternatively, the proteins are connected by a flexible polylinker such as Gly-Cys-Gly or Gly-Gly-Gly-Gly-Ser repeated 1 to 3 times (Bird et al., 1988, Science 242:423-426; Chaudhary et al., 1990, Proc. Nat'l. Acad. Sci. U.S.A. 87:1066-1070). In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (an *EL* coding sequence joined in-frame to a coding sequence for another antigen or a heterologous protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Chimeric genes comprising portions of the *EL* coding sequence fused to any other coding sequences may be constructed.

In another specific embodiment, the derivative is a molecule comprising a region of homology with EL. By way of example, in various embodiments, a protein region can be considered "homologous" to a second protein region when the amino acid sequence of the first region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, or 95% identical, when compared to any sequence in the second region of an equal number of amino acids as the number contained in the first region or when compared to an aligned sequence of the second region that has been aligned by a computer homology program known in the art.

5.3. PRODUCTION OF EL POLYPEPTIDES

In order to produce a biologically active EL, the nucleotide sequence coding for EL, or a functional equivalent, is inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The EL gene product as well as host cells or cell lines transfected or transformed with recombinant *EL* gene-containing expression vectors can be used for a variety of purposes. These include, but are not limited to, large scale production of EL protein, use of EL as immunogen for antibody generation and screening of compounds that bind EL.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the *EL* coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. (See, for example, the techniques described in Sambrook et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.). RNA capable of encoding EL polypeptide may also be chemically synthesized (Gait, ed., 1984, Oligonucleotide Synthesis, IRL Press, Oxford).

A variety of host-expression vector systems may be utilized to express the *EL* coding sequence. These include, but are not limited to, microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the *EL* coding sequence; yeast (e.g.,

Saccharomyces, *Pichia*) transformed with recombinant yeast expression vectors containing the *EL* coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the *EL* coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the *EL* coding sequence; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3 cells). The expression elements of these systems vary in their strength and specificities.

Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac hybrid promoter; cytomegalovirus promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedron promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll α/β binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the *EL* coding sequence, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

5.3.1. EXPRESSION SYSTEMS

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the expressed EL product. For example, when large quantities of EL protein are to be produced for the generation of antibodies, screening peptide libraries or formulating pharmaceutical compositions, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the *E. coli* expression vector pUR278

(Ruther et al., 1983, EMBO J. 2:1791), in which the *EL* coding sequence may be ligated into the vector in frame with the *lacZ* coding region so that a hybrid protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

In yeast, a number of vectors containing constitutive or inducible promoters may be used (Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II).

In cases where plant expression vectors are used, the expression of the *EL* coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. (Weissbach & Weissbach, 1988, Methods for Plant

Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9).

An alternative expression system which could be used to express *EL* is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The *EL* coding sequence may be cloned into non-essential regions (for example the polyhedron gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedron promoter). Successful insertion of the *EL* coding sequence will result in inactivation of the polyhedron gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedron gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (e.g., see Smith et al., 1983, J. Viol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the *EL* coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing EL in infected hosts (*e.g.*, See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may be used (See, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. USA 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. USA 79:4927-4931). Regulatable expression vectors such as the tetracycline repressible vectors may also be used to express the coding sequences in a controlled fashion.

Specific initiation signals may also be required for efficient translation of inserted *EL* coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire *EL* gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the *EL* coding sequence is inserted, exogenous translational control signals, including the ATG

initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the *EL* coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. The presence of several consensus N-glycosylation sites in the EL protein support the possibility that proper modification may be important for EL function. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, yolk sac cells, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the EL protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the *EL* coding sequence controlled by appropriate expression control elements (e.g., promoter and/or enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, genetically engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines

which express the EL protein. Such engineered cell lines are particularly useful in screening for molecules or drugs that affect EL function.

A number of selection systems may be used, including but not limited to, the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for *dhfr*, which confers resistance to methotrexate (Wigler, et al., 1980, Proc. Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and *hygro*, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Additional selectable genes include *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); *ODC* (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) and glutamine synthetase (Bebbington *et al.*, 1992, Biotech 10:169).

The expression characteristics of an endogenous *EL* gene within a cell line or microorganism may be modified by inserting a heterologous DNA regulatory element into the genome of a stable cell line or cloned microorganism such that the inserted regulatory element is operatively linked with the endogenous *EL* gene. For example, an endogenous *EL* gene which is normally "transcriptionally silent", *i.e.*, an *EL* gene which is normally not expressed, or is expressed only at very low levels in a cell line or microorganism, may be activated by inserting a regulatory element which is capable of promoting the expression of a normally expressed gene product in that cell line or microorganism. Alternatively, a transcriptionally silent, endogenous *EL* gene may be activated by insertion of a promiscuous regulatory element that works across cell types.

A heterologous regulatory element may be inserted into a stable cell line or cloned microorganism, such that it is operatively linked with an endogenous *EL* gene, using techniques, such as targeted homologous recombination, which are well known to those of skill in the art (*e.g.*, in Chappel, U.S. Patent No. 5,272,071; PCT publication No. WO 91/06667, published May 16, 1991).

5.3.2. PROTEIN PURIFICATION

Once a recombinant protein is expressed, it can be identified by assays based on the physical or functional properties of the product, including radioactive labeling of the product followed by analysis by gel electrophoresis, radioimmunoassay, ELISA, bioassays, etc.

Once the encoded protein is identified, it may be isolated and purified by standard methods including chromatography (*e.g.*, high performance liquid chromatography, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The actual conditions used will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity, etc., and will be apparent to those having skill in the art. The functional properties may be evaluated using any suitable assay such as lipase activities. For the practice of the present invention, it is preferred that the polypeptide is at least 80% purified from other proteins. It is more preferred that they are at least 90% purified. For *in vivo* administration, it is preferred that it is greater than 95% purified.

In another alternate embodiment, native proteins can be purified from natural sources, by standard methods such as those described above (*e.g.*, immunoaffinity purification). In a specific embodiment of the present invention, the EL polypeptides, whether produced by recombinant DNA techniques or by chemical synthetic methods or by purification from natural sources include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequences substantially as recited in SEQ ID NOS:2 or 3, as well as fragments and other derivatives, and analogs thereof, including proteins homologous thereto.

5.4. IDENTIFICATION OF CELLS THAT EXPRESS EL

The host cells which contain the coding sequence and which express an *EL* gene product, fragments thereof, or an EL fusion protein may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of *EL* mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity. Prior to the identification of gene expression, the host cells may be first mutagenized in an effort to increase the level of expression of *EL*, especially in cell lines that produce low amounts of EL.

In the first approach, the presence of the *EL* coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the *EL* coding sequence or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the *EL* coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the *EL* coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the *EL* coding sequence under the control of the same or different promoter used to control the expression of the *EL* coding sequence. Expression of the marker in response to induction or selection indicates expression of the *EL* coding sequence.

In the third approach, transcriptional activity for the *EL* coding region can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the *EL* coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes. Additionally, RT-PCR may be used to detect low levels of gene expression.

In the fourth approach, the expression of the EL protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmunoprecipitation, enzyme-linked immunoassays and the like. This can be achieved by using an anti-EL antibody. Alternatively, the phospholipase A1 activities of EL can be determined by assaying its ability to catalyze the breakdown of phospholipids.

5.5. ANTIBODIES TO EL AND THEIR USES

Antibodies directed to EL are useful for the identification and isolation of EL. In a preferred embodiment, an anti-EL antibody competitively inhibits EL protein and neutralize its activity. Alternatively, an anti-EL antibody may activate EL function. Anti-EL antibodies may be used in detecting and quantifying expression of EL levels in cells and tissues such as endothelial cells and certain tumor cells, as well as isolating EL-positive cells from a cell mixture or eliminating such cells by cytotoxicity.

Various procedures known in the art may be used for the production of antibodies to epitopes of the naturally-occurring, synthetic and recombinantly produced EL protein. Such antibodies include, but are not limited, to polyclonal, monoclonal, chimeric, humanized, single chain, anti-idiotypic, antigen-binding antibody fragments and fragments produced by a variable region expression library. Neutralizing antibodies i.e., those which compete for the substrate binding site of the EL protein are also encompassed by the invention.

Monoclonal antibodies that bind EL may be radioactively labeled allowing one to follow their location and distribution in the body after injection. Radioisotope tagged antibodies may be used as a non-invasive diagnostic tool for imaging *de novo* endothelial cells in tumors and metastases.

Immunotoxins may also be designed which target cytotoxic agents to specific sites in the body. For example, high affinity EL specific monoclonal antibodies may be covalently complexed to bacterial or plant toxins, such as diphtheria toxin or ricin. A general method of preparation of antibody/hybrid molecules may involve use of thiol-crosslinking reagents such as SPDP, which attack the primary amino groups on the antibody and by disulfide exchange, attach the toxin to the antibody. The hybrid antibodies may be used to specifically eliminate EL-expressing blood vessels in tumors.

For the production of antibodies, various host animals may be immunized by injection with the recombinant or naturally purified EL protein, fusion protein or peptides, including but not limited to rabbits, mice, rats, hamsters, and the like. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacilli Calmette-Guerin) and *Corynebacterium parvum*.

Monoclonal antibodies to EL may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including, but not limited to, IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the monoclonal antibodies of this invention may be cultivated *in vitro* or *in vivo*.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454; United States Patent Nos. 4,816,567 and 4,816,397) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Humanized antibodies may be generated according to the methods described in United States Patent Nos. 5,693,762; 5,585,089 and 5,565,332.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted

to produce single chain antibodies against gene products of interest. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibodies to the polypeptides of the invention can, in turn, be utilized to generate anti-idiotype antibodies that mimic an epitope of the polypeptide of interest, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). For example, antibodies which competitively inhibit the binding of an antibody to an antigenic peptide may mimic the antigenic epitope of the peptide. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used.

Hybridomas may be screened using enzyme-linked immunosorbent assays (ELISA) or radioimmunoassays in order to detect cultures secreting antibodies specific for refolded recombinant EL. Subsequent testing may use recombinant EL fragments to identify the specific portion of the EL molecule with which a monoclonal antibody binds. Additional testing may be used to identify monoclonal antibodies with desired functional characteristics such as staining of histological sections, immunoprecipitation or Western blotting of EL, or neutralization of EL activity. Determination of the monoclonal antibody isotype may be accomplished by ELISA, thus providing additional information concerning purification or function.

Antibody fragments which recognize specific binding sites of EL may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281; United States Patent Nos. 5,223,409; 5,403,484 and 5,571,698) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to EL. Antibody constant regions can be altered by molecular manipulations to modify their effector functions (United States Patent No. 5,624,821). The complementarity-determining regions (CDR) of an antibody can be identified, and synthetic peptides corresponding to such regions are used to mediate antigen binding (United States Patent No. 5,637,677).

5.6. USES OF GENETICALLY ENGINEERED HOST CELLS

In an embodiment of the invention, the EL protein and/or cell lines that express EL may be used to screen for antibodies, peptides, small molecules, natural and synthetic compounds or other cell bound or soluble molecules that bind to the EL protein, especially those that cause a stimulation or inhibition of EL function. For example, anti-EL antibodies may be used to inhibit or stimulate EL function and to detect its presence. Alternatively, screening of peptide libraries with recombinantly expressed soluble EL protein or cell lines expressing EL protein may be useful for identification of therapeutic molecules that function by inhibiting or stimulating the biological activities of EL. The uses of the EL protein and engineered cell lines, described in the sections below, may be employed equally well for homologous *EL* genes in various species.

In one embodiment of the invention, engineered cell lines which express the *EL* coding region or a portion of it that is fused to another molecule such as the immunoglobulin constant region (Hollenbaugh and Aruffo, 1992, Current Protocols in Immunology, Unit 10.19; Aruffo et al., 1990, Cell 61:1303) may be utilized to produce a soluble molecule with increased half life. The soluble protein or fusion protein may be used in binding assays, affinity chromatography, immunoprecipitation, Western blot, and the like. Synthetic compounds, natural products, and other sources of potentially biologically active materials can be screened in assays that are well known in the art.

Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to bind to EL, especially its active site (Lam, K.S. et al., 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that stimulate or inhibit the biological activities of EL.

Identification of molecules that are able to bind to the EL protein may be accomplished by screening a peptide library with recombinant soluble EL protein. Methods for expression and purification of EL are described in Section 5.3, *supra*, and may be used to express recombinant full length EL or fragments of EL depending on the functional domains of interest. EL may be used to identify a cofactor such as apolipoprotein.

To identify and isolate the peptide/solid phase support that interacts and forms a complex with EL, it may be necessary to label or "tag" the EL molecule. In addition, anti-EL antibody may be used to detect EL bound to a second molecule. The EL protein may be conjugated to enzymes such as alkaline phosphatase or horseradish peroxidase or to other reagents such as fluorescent labels which may include fluorescein isothiocyanate (FITC), phycoerythrin (PE) or rhodamine. Conjugation of any given label to EL may be performed using techniques that are well known in the art. Alternatively, *EL*-containing expression vectors may be engineered to express a chimeric EL protein containing an epitope for which a commercially available antibody exist. The epitope specific antibody may be tagged using methods well known in the art including labeling with enzymes, fluorescent dyes or colored or magnetic beads.

The "tagged" EL conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C to allow complex formation between EL and peptide species within the library. The library is then washed to remove any unbound protein. If EL has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing substrates for either alkaline phosphatase or peroxidase, for example, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diaminobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-EL complex changes color, and can be easily identified and isolated physically under a dissecting microscope with a micromanipulator. If a fluorescent tagged EL molecule has been used, complexes may be isolated by fluorescence activated sorting. If a chimeric EL protein expressing a heterologous epitope has been used, detection of the peptide/EL complex may be accomplished by using a labeled epitope specific antibody. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

In addition to using soluble EL molecules, it is possible to detect peptides that bind to cell-associated EL using intact cells. The use of intact cells is preferred for use with cell surface molecules. Methods for generating cell lines expressing EL are described in Section 5.3, *supra*. The cells used in this technique may be either live or fixed cells. The cells may be incubated with the random peptide library and bind to certain peptides in the library to

form a "rosette" between the target cells and the relevant solid phase support/peptide. The rosette can thereafter be isolated by differential centrifugation or removed physically under a dissecting microscope. Intracellular proteins can be accessed by treating the cells with detergent.

As an alternative to whole cell assays for membrane bound receptors or receptors that require the lipid domain of the cell membrane to be functional, EL molecules can be reconstituted into liposomes where label or "tag" can be attached.

5.7. USES OF *EL* POLYNUCLEOTIDE

Because of their central importance in lipid metabolism, and the critical link between lipid levels and cardiovascular disease, the proteins encoded by the lipase gene family have been extensively studied. Although the physiological site of action for LPL and HL is at the luminal surface of blood vessels, vascular endothelial cells do not synthesize either enzyme (Braun and Severson, 1992, Biochem J. 287:337-347). LPL can be taken up by endothelial cells and then be recycled to the cell surface (Goldberg, 1996, J. Lipid Res. 37:693-703). The cloning and characterization of *EL* provides the first evidence for lipase production directly by the endothelial cell. Production of a lipase in the vessel wall would provide a mechanism for local regulation of lipolytic activity, allowing the vessel to participate in lipid metabolic processes that are related to atherosclerosis and other vascular diseases, as well as those that are related to angiogenesis. In that connection, EL may function as secretory protein or cell membrane-associated protein.

Although the primary function of LPL is to hydrolyze triglycerides in lipoproteins at the surface of the endothelium, lipase activity and LPL gene expression have been identified within the blood vessel wall and linked to vascular disease processes. *In situ* hybridization studies have found LPL gene transcription in macrophages within the vessel wall, and LPL activity has been characterized in diseased blood vessels (O'Brien et al., 1994, Am. J. Pathol. 144:538-548). LPL in the vessel wall is thought to serve a bridging function between lipid and cell-surface proteoglycans, and to promote macromolecular aggregates by stimulating both matrix proteoglycan and lipoprotein binding. Also, LPL increases LDL retention within the blood vessel wall (Rutledge and Goldberg, 1994, J. Lipid Res. 35:1152-1160). In contrast to the many beneficial effects of LPL on lipoprotein metabolism in

circulating blood, a lipase expressed in the vessel wall may be an atherogenic factor (Rehier et al., 1993, Arterioscler. Thromb. 13:190-196; Rutledge et al., 1997, Circ. Res. 80:819-828). Thus, EL may be involved in the genesis and progression of atherosclerotic disease.

The very high level of *EL* expression in the decidua suggests that EL may play an important role in placental lipid metabolism. Fatty acids are required by the fetus, placenta, and fetal membranes for the synthesis of complex lipids, such as phospholipids, triacylglycerols and cholesterol esters. These lipids form cell membranes, are precursors for hormones, and may provide metabolic substrates. LPL activity facilitates the hydrolysis of free fatty acids from maternal VLDL-bound triglycerides. The majority of these released fatty acids are then transferred to the fetus by an unknown mechanism. There is, however, evidence for placental phospholipid transfer involving lipid breakdown and resynthesis (Biezenski, 1969, Am. J. Obstet. Gynecol. 104:1177-1189). In addition, recent studies suggest that cholesterol plays a crucial role in specific processes during mammalian embryonic development, including modification of the Hedgehog protein (Ferase and Herz, 1998, Trends Genet. 14:115-120). Thus, EL may be involved in the uptake of lipoproteins from circulating maternal blood into the fetal membranes.

Since *EL* message is expressed in placenta, testis and the corpus luteum, EL may also play a role in the uptake of lipoprotein-derived cholesterol into steroidogenic tissues to synthesize steroid hormones. These tissues include the placenta, ovary, corpus luteum and testis. The main phospholipase function of HL converts phospholipid-rich HDL_2 to HDL_3. Hydrolysis of HDL_2 phospholipids may facilitate transfer of core cholesterol esters to cells which synthesize steroid hormones (Wang et al., 1996, J. Biol. Chem. 271:21001-21004).

EL mRNA was also detected in mouse lung tissue. The punctate hybridization pattern was not consistent with type I epithelial cell or endothelial cell *EL* expression, but was highly suggestive of *EL* expression by type II epithelial cells or macrophages. Alveolar type II epithelial cells synthesize surfactant which maintains alveolar patency (Rooney, 1985, Am. Rev. Respir. Dis. 131: 439-460). Disaturated phosphatidylcholine (DSPC), which is synthesized from long chain fatty acids, is the major lipid component of surfactant (Batenburg, 1992, Am. J. Physiol. 262:L367-385.). Surfactant synthesis is critically dependent on the availability of free fatty acids. One source of these lipids may be VLDL-

transported triglycerides, which are hydrolyzed locally by lipases to free fatty acids (Mallampalli et al., 1997, J. Clin. Invest. 99:2020-2029)). EL may play an integral role in providing fatty acid or lysophospholipid substrates for surfactant phospholipid synthesis by alveolar type II epithelial cells.

An *EL* polynucleotide may be used for diagnostic and/or therapeutic purposes. In addition, since EL is expressed in endothelial cells, particularly cells undergoing angiogenesis, an *EL* polynucleotide may be used to detect the expression of EL as an endothelial cell marker. For diagnostic purposes, an *EL* polynucleotide may be used to detect the level of *EL* gene expression, aberrant *EL* gene expression or mutations in disease states. Included in the scope of the invention are oligonucleotides such as antisense RNA and DNA molecules, and ribozymes, that function to inhibit translation of EL. An *EL* polynucleotide may also be used to construct transgenic and knockout animals for studying EL function *in vivo* and for the screening of EL agonists and antagonists in an animal model.

5.7.1. TRANSGENIC AND KNOCKOUT ANIMALS

The *EL* gene products can be expressed in animals by transgenic technology. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, sheep, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate *EL* transgenic animals. The term "transgenic," as used herein, refers to animals expressing *EL* coding sequences from a different species (*e.g.*, mice expressing human *EL* gene sequences), as well as animals that have been genetically engineered to overexpress endogenous (*i.e.*, same species) *EL* sequences or animals that have been genetically engineered to no longer express endogenous *EL* gene sequences (*i.e.*, "knock-out" animals), and their progeny.

Any technique known in the art may be used to introduce an *EL* transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Hoppe and Wagner, 1989, U.S. Patent No. 4,873,191); retrovirus-mediated gene transfer into germ lines (Van der Putten, *et al.*, 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson, *et al.*, 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol.

Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell 57:717-723) (see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115, 171-229).

Any technique known in the art may be used to produce transgenic animal clones containing an *EL* transgene, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal or adult cells induced to quiescence (Campbell, *et al.*, 1996, Nature 380:64-66; Wilmut, *et al.*, Nature 385:810-813).

The present invention provides for transgenic animals that carry an *EL* transgene in all their cells, as well as animals that carry the transgene in some, but not all their cells, *i.e.*, mosaic animals. The transgene may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.* (1992, Proc. Natl. Acad. Sci. USA 89:6232-6236). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the *EL* transgene be integrated into the chromosomal site of the endogenous *EL* gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous *EL* gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous *EL* gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous *EL* gene in only that cell type, by following, for example, the teaching of Gu, *et al.* (1994, Science 265: 103-106). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant *EL* gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques that include, but are not limited to, Northern blot analysis of tissue samples obtained from the

animal, *in situ* hybridization analysis, and RT-PCR. Samples of *EL* gene-expressing tissue may also be evaluated immunocytochemically using antibodies specific for the *EL* transgene product.

5.7.2. DIAGNOSTIC USES OF *EL* POLYNUCLEOTIDE

An *EL* polynucleotide may have a number of uses for the diagnosis of diseases resulting from aberrant expression of *EL*. Alternatively, polymorphisms or mutations may be identified in an *EL* nucleotide sequence which may be correlative with disease. For example, the *EL* nucleotide sequence or portions thereof may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of *EL* expression; *e.g.*, Southern analysis, Northern analysis, *in situ* hybridization assays and PCR. For PCR, primers of 15-25 nucleotides designed from any portion of *EL* nucleotide sequence are preferred. However, the length of primers may be adjusted by one skilled in the art. Such techniques are well known in the art, and are in fact the basis of many commercially available diagnostic kits. In cases of hypertriglyceridemia, detection of decreased *EL* expression or a mutation in *EL* may be used to determine an underlying cause of the condition, and facilitate treatment of the disease. Alternatively, in situations where EL is atherogenic, detection of its presence or overexpression may be used to predict development of vascular diseases and provide appropriate preventive measures.

5.7.3. THERAPEUTIC USES OF *EL* POLYNUCLEOTIDE

An *EL* polynucleotide may be useful in the treatment of various abnormal conditions. By introducing gene sequences into cells, gene therapy can be used to treat conditions in which the cells do not express normal *EL* or express abnormal/inactive *EL*. In some instances, the polynucleotide encoding *EL* is intended to replace or act in the place of a functionally deficient endogenous gene. Alternatively, abnormal conditions characterized by overexpression can be treated using the gene therapy techniques described below.

In a specific embodiment, nucleic acids comprising a sequence encoding an EL protein or a functional derivative thereof, are administered to promote EL function, by way

of gene therapy. Gene therapy refers to therapy performed by the administration of a nucleic acid to a subject. In this embodiment of the invention, the nucleic acid produces its encoded protein that mediates a therapeutic effect by promoting EL function. Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred embodiment of the invention, the therapeutic composition comprises an *EL* coding sequence that is part of an expression vector. In particular, such a nucleic acid has a promoter operably linked to the *EL* coding sequence, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another specific embodiment, a nucleic acid molecule is used in which the *EL* coding sequence and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the *EL* nucleic acid (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression

vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), by direct injection of naked DNA, by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), by coating with lipids or cell-surface receptors or transfecting agents, by encapsulation in liposomes, microparticles, or microcapsules, by administering it in linkage to a peptide which is known to enter the nucleus, or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) which can be used to target cell types specifically expressing the receptors. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180 dated April 16, 1992; WO 92/22635 dated December 23, 1992; WO92/20316 dated November 26, 1992; WO93/14188 dated July 22, 1993; WO 93/20221 dated October 14, 1993). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

In a preferred embodiment of the invention, adenoviruses as viral vectors can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells (Kozarsky and Wilson, 1993, Current Opinion in Genetics and Development 3:499-503). Bout et al., (1994, Human Gene Therapy 5:3-10) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., 1991, Science 252:431-434; Rosenfeld et al., 1992, Cell 68:143-155; and Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234. Adeno-associated virus (AAV) has

also been proposed for use in gene therapy (Walsh et al., 1993, Proc. Soc. Exp. Biol. Med. 204:289-300).

In addition, retroviral vectors (see Miller et al., 1993, Meth. Enzymol. 217:581-599) have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The *EL* coding sequence to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., 1994, Biotherapy 6:291-302, which describes the use of a retroviral vector to deliver the *mdr1* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., 1994, J. Clin. Invest. 93:644-651; Kiem et al., 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Another approach to gene therapy involves transferring a gene to cells in tissue culture. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, lipofection, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. In a preferred embodiment, endothelial cells are injected, *e.g.*, subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin graft onto the patient. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include, but are not limited, to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc. In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding sequence, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

Oligonucleotides such as anti-sense RNA and DNA molecules, and ribozymes that function to inhibit the translation of a *EL* mRNA are also within the scope of the invention. Such molecules are useful in cases where downregulation of *EL* expression is desired. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, *e.g.*, between -10 and +10 regions of a *EL* nucleotide sequence, are preferred.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine,

2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of *EL* RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Endogenous target gene expression can also be reduced by inactivating or "knocking out" the target gene or its promoter using targeted homologous recombination (*e.g.*, see Smithies, *et al.*, 1985, Nature 317:230-234; Thomas and Capecchi, 1987, Cell 51:503-512; Thompson, *et al.*, 1989, Cell 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional target gene (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous target gene (either the coding regions or regulatory regions of the target gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous

recombination, results in inactivation of the target gene. Such approaches are particularly suited in experiments where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive target gene (*e.g.*, see Thomas and Capecchi, 1987 and Thompson, 1989, *supra*). However, this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

Alternatively, endogenous target gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene (*i.e.*, the target gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the target gene in target cells in the body (See generally, Helene, 1991, Anticancer Drug Des., 6(6):569-584; Helene, *et al.*, 1992, Ann. N.Y. Acad. Sci., 660:27-36; and Maher, 1992, Bioassays 14(12):807-815).

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

The anti-sense RNA and DNA molecules, ribozymes and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which contain suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

Methods for introducing polynucleotides into such cells or tissues include methods for *in vitro* introduction of polynucleotides such as the insertion of naked polynucleotide, *i.e.*, by injection into tissue, the introduction of a *EL* polynucleotide in a cell *ex vivo*, the use of a vector such as a virus, (retrovirus, adenovirus, adeno-associated virus, etc.), phage or plasmid, etc. or techniques such as electroporation or calcium phosphate precipitation.

5.8. USES OF EL PROTEIN

The *EL* gene is upregulated in endothelial cells undergoing angiogenesis. Angiogenesis refers to the development of blood vessels from pre-existing vessels. Angiogenesis occurs throughout adult life as a wound healing response or to increase oxygenation of chronically stressed tissues (Pardanaud et al., 1989 *Development* 105:473; Granger 1994, *Cell and Mol. Biol. Res.* 40:81).

EL may play a functional role during angiogenesis. For therapeutic use, it is essential that EL, portions of EL or antibodies that block EL, interact with angiogenic cells since it is stimulation or inhibition of these cells that is clinically relevant.

EL protein inhibitors or anti-EL antibodies may function to directly interfere with EL enzymatic activities. In addition, they may be used to suppress angiogenesis or induce endothelial cell apoptosis. This function could be clinically useful to prevent neovascularization of tissues such as tumor nodules. It has been demonstrated that inhibition of angiogenesis is useful in preventing tumor metastases (Fidler and Ellis, 1994, *Cell* 79:185). O'Reilly et al (1994, *Cell* 79:315) reported that a novel angiogenesis inhibitor isolated from tumor-bearing mice, angiostatin, specifically inhibited endothelial cell proliferation. *In vivo*, angiostatin was a potent inhibitor of neovascularization and growth of tumor metastases.

The expression of EL or the modulation of its activation in endothelial cells may also be used to treat abnormal conditions that result from altered LPL activities. These conditions include, but are not limited to, hypertriglyceridemia, diabetes and renal failure. Additionally, the ability to increase expression of EL in endothelial cells may be used to promote angiogenesis. These conditions include, but are not limited to, cardiac ischemia, thrombotic stroke, would healing and peripheral vascular disease. Expression or activities of EL may be upregulated or downregulated depending on the desired outcome.

5.8.1 FORMULATION AND ROUTE OF ADMINISTRATION

A EL polypeptide, a fragment thereof or an anti-EL antibody may be administered to a subject *per se* or in the form of a pharmaceutical or therapeutic composition. Pharmaceutical compositions comprising the proteins of the invention may be manufactured by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries which facilitate processing of the protein or active peptides into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For topical administration the proteins of the invention may be formulated as solutions, gels, ointments, creams, suspensions, etc. as are well-known in the art.

Systemic formulations include those designed for administration by injection, *e.g.* subcutaneous, intravenous, intramuscular, intrathecal or intraperitoneal injection, as well as those designed for transdermal, transmucosal, oral or pulmonary administration.

For injection, the proteins of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution. Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the proteins may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, a composition can be readily formulated by combining the proteins with pharmaceutically acceptable carriers well known in the art. Such carriers enable the proteins to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. For oral solid formulations such as, for example, powders, capsules and tablets, suitable excipients include fillers such as sugars, such as lactose, sucrose, mannitol and sorbitol; cellulose preparations such as maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP); granulating agents; and binding agents. If desired, disintegrating agents may be added, such as the cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

If desired, solid dosage forms may be sugar-coated or enteric-coated using standard techniques.

For oral liquid preparations such as, for example, suspensions, elixirs and solutions, suitable carriers, excipients or diluents include water, glycols, oils, alcohols, etc. Additionally, flavoring agents, preservatives, coloring agents and the like may be added.

For buccal administration, the proteins may take the form of tablets, lozenges, etc. formulated in conventional manner.

For administration by inhalation, the proteins for use according to the present invention are conveniently delivered in the form of an aerosol spray from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The proteins may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, e.g, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the proteins may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the proteins may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Alternatively, other pharmaceutical delivery systems may be employed. Liposomes and emulsions are well known examples of delivery vehicles that may be used to deliver the proteins or peptides of the invention. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the proteins may be delivered using a sustained-release system, such as semipermeable matrices of solid polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the proteins for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

As the proteins of the invention may contain charged side chains or termini, they may be included in any of the above-described formulations as the free acids or bases or as pharmaceutically acceptable salts. Pharmaceutically acceptable salts are those salts which

substantially retain the biologic activity of the free bases and which are prepared by reaction with inorganic acids. Pharmaceutical salts tend to be more soluble in aqueous and other protic solvents than are the corresponding free base forms.

5.8.2. EFFECTIVE DOSAGES

EL polypeptides, EL fragments and anti-EL antibodies will generally be used in an amount effective to achieve the intended purpose. The proteins of the invention, or pharmaceutical compositions thereof, are administered or applied in a therapeutically effective amount. By therapeutically effective amount is meant an amount effective ameliorate or prevent the symptoms, or prolong the survival of, the patient being treated. Determination of a therapeutically effective amount is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure provided herein.

For systemic administration, a therapeutically effective dose can be estimated initially from *in vitro* assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

Initial dosages can also be estimated from *in vivo* data, *e.g.*, animal models, using techniques that are well known in the art. One having ordinary skill in the art could readily optimize administration to humans based on animal data.

Dosage amount and interval may be adjusted individually to provide plasma levels of the proteins which are sufficient to maintain therapeutic effect. Usual patient dosages for administration by injection range from about 0.1 to 5 mg/kg/day, preferably from about 0.5 to 1 mg/kg/day. Therapeutically effective serum levels may be achieved by administering multiple doses each day.

In cases of local administration or selective uptake, the effective local concentration of the proteins may not be related to plasma concentration. One having skill in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

The amount of EL administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

The therapy may be repeated intermittently while symptoms detectable or even when they are not detectable. The therapy may be provided alone or in combination with other drugs. In the case of hypercholesterolemia, other conventional drugs may be used in combination with EL or fragments thereof.

5.8.3. TOXICITY

Preferably, a therapeutically effective dose of the proteins described herein will provide therapeutic benefit without causing substantial toxicity.

Toxicity of the proteins described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, by determining the LD_{50} (the dose lethal to 50% of the population) or the LD_{100} (the dose lethal to 100% of the population). The dose ratio between toxic and therapeutic effect is the therapeutic index. The data obtained from these cell culture assays and animal studies can be used in formulating a dosage range that is not toxic for use in human. The dosage of the proteins described herein lies preferably within a range of circulating concentrations that include the effective dose with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (*See, e.g.*, Fingl *et al.*, 1975, In: The Pharmacological Basis of Therapeutics, Ch.1, p.1).

The invention having been described, the following examples are offered by way of illustration and not limitation.

6. EXAMPLE: MOLECULAR CLONING OF A NOVEL LIPASE CODING SEQUENCE FROM HUMAN ENDOTHELIAL CELLS

6.1. MATERIALS AND METHODS

6.1.1. CELL CULTURES

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) and cultured in media (EGM) supplied by the company supplemented with 15% fetal calf serum (FCS). Approximately 480 µl of "MATRIGEL" (Collaborative

Biomedical Products, Bedford, MA), an extract containing basement membrane-like components, were applied to 35 mm tissue culture dishes. HUVEC (3.9×10^4 eell/cm^2) were plated onto the geLand incubated at 37°C for 3 hours. After incubation, HUVEC plated on "MATRIGEL" elongated and migrated to form a network-like structure. The culture medium was removed and then the adherent layer of cells on the "MATRIGEL" washed three times with cold PBS. MatriSperse solution (Collaborative Biomedical Products) was added at 2 ml per 35 mm dish, and the cell/gel layer was scraped and transferred into 50 ml conical tubes. The dish was rinsed with 2 ml of MatriSperse solution and the solution was also transferred into the tube. After "MATRIGEL" was dissolved at 4°C for 1 hour and HUVEC were released from the gel, HUVEC were washed with PBS(-) three times. These HUVEC were used for isolation of mRNA by MicroFast Track kit (Invitrogen, Carlsbad, CA).

COS-7 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in DMEM containing 10% FCS.

6.1.2. CLONING BY SUPPRESSION SUBTRACTIVE HYBRIDIZATION

A polymerase chain reaction (PCR)-based cDNA subtraction method termed suppression subtractive hybridization (Clontech, Palo Alto, CA) was employed to identify genes preferentially expressed in tube-forming endothelial cells. First, cDNA was synthesized from RNA of tube-forming ("MATRIGEL"-treated) HUVEC, and growth arrested HUVEC. Tester DNA was derived from 2 μg of tube-forming HUVEC PolyA (+) RNA, and driver DNA was derived from 2 μg of growth arrested and cobblestone-like HUVEC polyA (+) RNA. The tester and driver DNA were digested with *Rsa*I that yielded blunt ends. The tester DNA was then divided into two portions and each ligated with a different cDNA adapter, and two hybridizations were performed. In the first, an excess of driver was added to each sample of tester. The sample was then heat denatured and allowed to anneal. During the second hybridization, the two primary hybridization samples were mixed together without denaturing. After filling in the ends by DNA polymerase, the entire population of molecules was then subjected to PCR to amplify the differentially expressed

sequences in the tube-forming ("MATRIGEN"-treated) HUVEC. Products from secondary PCRs were inserted into pT7-Blue T vectors (Novagen, Madison, WI) and sequenced by dideoxy method. Nucleic acid homology searches were performed using the BLAST program at the National Center for Biotechnology Information. DNA and protein sequence analyses were performed using the Wisconsin Package software from Genetics Computer Group, Inc. (Madison, WI).

6.1.3. SCREENING OF cDNA LIBRARY

λgt 11 5'-stretch mouse 11 day embryo and λgt 10 HUVEC cDNA libraries (Clontech, Palo Alto, CA) were used for screening. The HUVEC cDNA library was screened with a [^{32}P]-labeled dCTP probe. These cDNA probes were radiolabeled by a random prime labeling method. Hybridization of the cDNA libraries was performed according to standard protocols.

6.1.4. 5' RAPID AMPLIFICATION OF cDNA ENDS

A 5'RACE was performed according to the manufacturer's instructions (GIBCO BRL, Gaithersburg, MD). By antisense primer 5'-GATCAAGTGGACATTCC-3' (SEQ ID NO:6), single stranded cDNA was prepared from human placenta poly (A)+RNA. An anchor primer was then added to the cDNA pool 3' end with terminal deoxynucleotide transferase and dCTP. PCR was carried out using this modified cDNA template, an anchor primer, and antisense primer 5'-GTCCTTCTCCTGCAGCCAGTCG-3' (SEQ ID NO:7). Thermocycling was performed for 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min. The resulting products were cloned directly into PT7-Blue T vectors (Novagen, Madison, WI).

6.2. RESULTS

Suppression subtractive hybridization was used to isolate cDNA clones preferentially expressed in endothelial cells undergoing tube formation on ""MATRIGEL"" as a model of angiogenesis. A total 248 clones were sequenced and analyzed. Nucleotide sequence analysis revealed that 37.5% of the isolated genes (93/248) had not been

previously characterized, and 62.5% of isolated genes (155/248) had been characterized. Seventy five of 93 uncharacterized genes (81%) matched sequences in expressed sequence tag (EST) database. Nine clones were chosen as candidates of genes specifically expressed in endothelial cells.

Northern blot analysis revealed that mRNA expression of certain clones were upregulated in tube-forming HUVEC plated on "MATRIGEL" compared to that in non-dividing cobblestone-like HUVEC, while other mRNA expression was decreased in HUVEC cultured on "MATRIGEL". In order to obtain the entire open reading frame of these clones, an oligo-dT primed λgt10 HUVEC cDNA library was screened and 5'-RACE was employed. The longest clones were subjected to nucleotide sequence analysis and database searching employing BLAST. This analysis resulted in the identification of one clone, designated C18. The C18 coding sequence is expressed in human endothelial cells, and its expression is upregulated in the cells undergoing differentiation or angiogenesis.

Figure 1A-1C discloses the nucleotide sequence (SEQ ID NO:1) and the deduced amino acid sequence (SEQ ID NO:2) of C18. The initiating methionine is located at amino acid position #1. The signal peptide is predicted to be cleaved between amino acid position #18 and #19. A translation stop codon is found at amino acid position #501.

This clone shared sequence homology with LPL, as shown by 44% identity between the C18 amino acid sequence and a human LPL (Wion *et al.*, 1987, Science 235:1638) (Figure 2). Since there was no prior evidence that a lipase was produced by an endothelial cell, the C18 gene product is herein referred to as endothelial cell lipase (EL). Interestingly, EL was found to share the highest identity with a chicken LPL amino acid sequence at 49% (Cooper *et al.*, 1989, Biochimica et Biophysica Acta 1008:92).

Murine *EL* mRNA was characterized through screening an 11-day embryonic λgt11 cDNA library with a human cDNA probe. The entire homologous murine EL protein was encoded by a 2272 bp clone. The open reading frames of both the human and mouse EL coding sequences were 1500 nucleotides, encoding highly conserved proteins of 500 amino acids (Figure 2). A hydrophobicity plot analysis (Kyte and Doolittle, 1982, J. Mol. Biol. 157:105-132) of the predicted amino acid sequence revealed a hydrophobic leader peptide with a putative signal cleavage site located 18 amino acids downstream of the translation

initiation site in both proteins. Both mature human and mouse EL proteins consist of 482 amino acids. EL shares sequence homology with the mammalian lipases including 44% amino acid identity to LPL and 34% amino acid identity to hepatic lipase (HL). Alignment with the human LPL and HL amino acid sequences indicates conservation of the catalytic residues serine (Ser 169), aspartic acid (Asp 193) and histidine (His 274) as well as ten cysteine residues involved in disulfide bridge formation.

Similarly, two stretches of hydrophobic amino acids (163-172 and 272-281) that are adjacent to the catalytic serine and histidine, respectively, and believed to be important for EL interaction with lipid substrate are also conserved. In addition, like LPL and HL, EL possesses a lid consisting of 19 amino acids. By comparing with the predicted three dimensional structures of LPL and HL, the EL lid covers a catalytic pocket, and is probably repositioned to allow substrates to come into contact with the catalytic residues. The EL lid displays minimal sequence identity to the lids of LPL and HL, but the regions bordering the lid are almost identical among EL, LPL and HL. The lid structure has been demonstrated to confer substrate specificities for LPL and HL. In addition, alignment with the human LPL sequence indicates conservation of positively charged clusters involved in heparin binding. These clusters in EL include cluster 1: Arg 327-Lys 329-Arg 330-Lys 333; cluster 2: Arg 312-Lys 313-Arg 315 and cluster 4: Lys 352-Arg 450-Lys 452-Lys 459. Moreover, five potential glycosylation sites are predicted by the presence of the universal acceptor sequence Asn-X-(Thr-Ser) at positions 80, 136, 393, 469, and 491. These glycosylation sites may modulate the heparin binding properties of EL.

7. EXAMPLE: TISSUE DISTRIBUTION OF *EL* EXPRESSION

7.1. MATERIALS AND METHODS

7.1.1. NORTHERN BLOT ANALYSIS

In order to study the expression pattern of the *EL* gene, Northern blots containing RNA obtained from a variety of human and mouse tissues (Clontech, Palo Alto, CA) were hybridized with one of two *EL* probes: a probe corresponding to a 514 base pair human cDNA fragment from nucleotide #314 through #828 of SEQ ID NO:1, and a probe corresponding to the entire mouse cDNA clone (SEQ ID NO:5). The hybridization and

wash conditions for the ExpressHyb buffer (Clontech) were used in accordance with the manufacturer's recommendation. Briefly, the blots were prehybridized at 65°C for 1 hour in a solution containing ExpressHyb. The probes were labeled with [^{32}P]dCTP by random priming (Stratagene, La Jolla, CA), heat denatured and added to the prehybridization mix at 42°C for 16-24 hours in the presence of 40% formamide and 10% dextran sulfate. The blots were washed by the following conditions: 65°C in 2X SSC buffer and 0.5% SDS several times before being transferred to a wash solution containing 0.1X SSC, 0.1% SDS and agitated at 50°C for 40 minutes. The blots were then covered with plastic wrap, mounted on Whatman paper and exposed to Kodak Biomax Ms x-ray film (Eastman Kodak, Rochester, NY) at -70°C overnight using an intensifying screen.

7.1.2. *IN SITU* HYBRIDIZATION

In situ hybridization slides were generated from paraformaldehyde-fixed, paraffin-embedded mouse embryos according to established methodology or were purchased from Novagen (Madison, WI). A 611-bp *Eco*IRI mouse *EL* cDNA fragment encoding the carboxyl terminus 52 amino acids and 3' untranslated region was cloned into pBluescript KS(+). This fragment was used for *in vitro* RNA probe transcription. Both antisense and sense cRNA probes were labeled with [^{35}S]dUTP. Hybridization, washing, and probe detection were performed as described by Hogan et al.

7.2. RESULTS

Northern blot analysis of adult human tissues revealed an intense band representing a 4.4 Kb mRNA in placenta and liver (Figure 3A). A weaker *EL* signal was detected in the brain and kidney. In addition, expression of a same size message was also detected in adult mouse tissues, including lung, liver and testis (Figure 3B). Weaker signals were observed in the murine heart and kidney. A strong signal was also detected in the thyroid. When embryonic tissues were examined, an intense signal was detected in a 7-day mouse embryo, likely due to its expression in the placental tissue which was included in RNA isolation (Figure 4B). Week signals were detected in 11, 15 and 17-day mouse embryos. In human embryos, *EL* expression was detected in the brain, lung, liver and kidney (Figure 4A).

In order to more closely examine the cell types that expressed *EL*, *in situ* hybridization of embryonic and adult murine tissue sections was performed using ^{35}S-labeled cRNA probes. An intense hybridization signal over the decidua surrounding mouse embryos was consistent with *EL* expression in trophoblast cells. Extensive experiments with early and mid-gestation embryos revealed no specific hybridization pattern above the background signal observed with the sense probe. In the adult, expression of *EL* in the lung was visualized as a punctate pattern in the pulmonary alveoli. This pattern was consistent with expression of *EL* in either type II alveolar cells or macrophages. A strong signal was also observed in the corpus luteum of the ovary. *In situ* hybridization of liver revealed low level expression by all cell types.

8. EXAMPLE: EL EXHIBITS LIPASE ACTIVITIES

8.1. MATERIALS AND METHODS

8.1.1. RECOMBINANT EL PRODUCTION

In order to express a recombinant human EL, an in-frame coding sequence was ligated to a coding sequence for a carboxyl-terminal c-Myc-peptide: EQKLISEED (SEQ ID NO:8). The following primers were utilized in PCR:

5'-GGCTCGAGCCACCATGAGCAACTCCGTTCCTCTGCTCTGT-3 (SEQ ID NO:9), and

5'-GGCTCGAGCTACAGATCTTCTTCAGAAATAAGTTTTTGTTCGGGAAGCTCCAC AGTGG GACT-3 (SEQ ID NO:10). This hybrid DNA fragment was then cloned into the eukaryotic expression vector phbAPr-3-neo. Human EL-c-myc expressing plasmids were subsequently transfected into COS-7 cells with Lipofectamine (GIBCO BRL, Gaithersburg, MD). The cells were selected in the presence of 1000 μg/ml of G418 (GIBCO BRL, Gaithersburg, MD). Expression levels of human EL protein were determined by Western blotting using anti-c-Myc monoclonal antibodies (Boehringer Mannheim, Indianapolis, IN). The clones which synthesized c-Myc-tagged EL proteins were selected for further study. Negative control clones were randomly selected from a transfection with phbAPr-3-neo vector alone.

8.1.2. LIPASE ACTIVITY ASSAY

COS-7 cells stably expressed both human EL phbAP-3-neo and c-myc/phbAP-3-neo vectors. The COS-7 cells were cultured in serum-free DMEM with two units of heparin. After 36 h, the conditioned media was harvested, and was concentrated from 1 to 10 ml by Centricon 10 (Millipore, Bedford, MA). After glycerol was added to a 30% final concentration, the conditioned medium was stored at -80°C. The cells were harvested by scraping into lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCℓ, 1% CHAPS, 10 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, 1 mM PMSF).

Esterase activity and triglyceride lipase activity were quantitated in triplicate by [^{14}C]tributyrin (Shirai et al., 1984, Biochem Biophys Acta 795) and [^{14}C]triolein activity assays (Iverius and Brunzell, 1985, Am. J. Physiol. 249:E107-114). Phospholipase activity was measured using phospholipid vesicles. The phospholipid vesicles were synthesized using a modification of the triolein emulsion: 20 mg/ml dioleoylphosphatidylcholine (DOPC) (Sigma, St. Louis, MO) was used in place of egg yolk extract. Labeled triolein was substituted with 1,2 di [1-^{14}C] L-3-phosphatidylcholine (Amersham Life Science, Arlington Heights, IL) at an activity of 0.06 μCi/ml of substrate. Substrate (200 μl) was added to 100 μl of medium from transfected cells in a final volume of 330 μl of 150 mM NaCl, 100mM Tris-HCl, pH 8.5, 2.5% bovine serum albumin, 2 units/ml heparin (Elkins-Sinn, Cherry Hill, NJ) in the presence or absence of 10 μl of human plasma as source of apoC-II. The samples were incubated at 37°C for 2 h followed by oleic acid extraction (Belfrage and Vaughan, 1969, J. Lipid Res. 10:341-344) and scintillation counting.

8.2. RESULTS

Recombinant EL fusion protein was expressed in eukaryotic cells and tested for functional activities. An expression construct encoding a c-Myc-tagged human EL fusion protein, EL-c-Myc/phbAPr-3-neo, was transfected into COS-7 cells. By western blot analysis, greater than 95% of the EL-c-Myc-tagged protein was secreted into the culture supernatant. The presence of esterase activity of the *EL-c-Myc* fusion protein was established using the short chain, water soluble substrate tributyrin, while its triglyceride lipase activity was tested using the long chain, lipid substrate triolein. Phospholipase A1

activities of the EL-c-Myc were determined in culture supernatants and compared with supernatants from cells transfected with vector alone (Table I). Since EL shares sequence identity with LPL, and LPL activity is enhanced in the presence of apo-CII, the functional activity of EL was also tested in the presence of apo-CII. The phospholipase A1 activity of EL was approximately twice that of vector alone (307±25 versus 156±34 nmol FFA/ml/h, $p<0.00005$). Interestingly, this phospholipase A1 activity was partially inhibited by apo-CII. However, EL-c-Myc showed no triglyceride lipase activity in the presence or absence of apo-CII.

Table I. Phospholipase Activity of Human Endothelial Cell Lipase.

	apo-CII(-)	apo-CII(+)
EL-c-Myc	307±25*	249±32
Vector alone	156±34*	174±5
Corrected c-Myc-EL	151±25+	75±32

- *$p<0.00005$ compared to vector alone.
- +$p<0.04$ compared to activity in the presence of apo-CII

* Phospholipase activity was measured in the culture supernatant of transfected COS-7 cells. Lipase activity is expressed as nmol FFA/ml/h.

The present invention is not to be limited in scope by the exemplified embodiments or deposited organisms which are intended as illustrations of single aspects of the invention, and any clones, DNA or amino acid sequences which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims. It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purposes of description.

All publications cited herein are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide, comprising a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence as shown in SEQ ID NO:2.

2. The polynucleotide of Claim 1 in which the nucleotide sequence is shown in SEQ ID NO:1.

3. An isolated polynucleotide, comprising a nucleotide sequence of at least 12 nucleotides that hybridizes under stringent conditions to a second polynucleotide having a nucleotide sequence as shown in SEQ ID NO:1 or to the complementary sequence of the second polynucleotide.

4. The polynucleotide of Claim 3 which encodes a lipase.

5. An isolated polynucleotide, comprising a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence as shown in SEQ ID NO:3.

6. The polynucleotide of Claim 5 in which the nucleotide sequence is shown in SEQ ID NO:5.

7. An isolated polynucleotide, comprising a nucleotide sequence of at least 12 nucleotides that hybridizes under stringent conditions to a second polynucleotide having a nucleotide sequence as shown in SEQ ID NO:5 or to the complementary sequence of the second polynucleotide.

8. The polynucleotide of Claim 7 which encodes a lipase.

9. The isolated polynucleotide of Claim 1 which is DNA.

10. The isolated polynucleotide of Claim 9 which is cDNA.

11. A recombinant vector containing the polynucleotide of Claim 1.

12. A recombinant expression vector containing the polynucleotide of Claim 1 in which the nucleotide sequence is operatively associated with a regulatory nucleotide sequence that controls expression of the polynucleotide in a host cell.

13. A genetically-engineered host cell containing the expression vector of claim 12, or progeny thereof.

14. The genetically-engineered host cell of claim 13 which is a prokaryote.

15. The genetically-engineered host cell of claim 13 which is an eukaryote.

16. A method for producing a polypeptide, comprising:

(a) culturing the genetically-engineered host cell of Claim 14; and

(b) recovering the polypeptide from the cultured host cell or its culture medium.

17. A method for producing a polypeptide, comprising:

(a) culturing the genetically-engineered host cell of Claim 15; and

(b) recovering the polypeptide from the cultured host cell or its culture medium.

18. An isolated polypeptide comprising the amino acid sequence as shown in SEQ ID NO:2.

19. An isolated polypeptide comprising the amino acid sequence as shown in SEQ ID NO:3.

20. An isolated polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a second polynucleotide comprising a complementary nucleotide sequence that encodes the amino acid sequence as shown in SEQ ID NO:2.

21. The polypeptide of Claim 18 which is produced by a recombinant DNA method.

22. The polypeptide of Claim 18 which is fused with a heterologous peptide.

23. A pharmaceutical composition comprising the polypeptide of Claim 18 and a pharmaceutically acceptable carrier.

24. An antibody which specifically binds to the polypeptide of Claim18, or an antigen-binding fragment thereof.

25. The antibody of Claim 24 which is a monoclonal antibody.

26. A method for diagnosing hypertriglyceridemia, comprising detecting decreased expression of the polynucleotide of Claim 1 in a cell.

27. A method for diagnosing hypercholesterolemia, comprising detecting increased expression of the polynucleotide of Claim 1 in a cell.

28. A method for treating hypertriglyceridemia, comprising increasing the expression of the polypeptide of Claim 18.

29. A method for treating hypercholesterolemia, comprising decreasing the expression of the polypeptide of Claim 18.

30. A method for treating cancer, comprising decreasing the expression of the polypeptide of Claim 18.

```
AGCAGCGAGTCCTTGCTTCCCGGCGGCTCAGGACGAGGGCAGATCTCGTTCTGGGGC

         10         20         30         40         50

AAGCCGTTGACACTCGCTCCCTGCCACCGCCCGGGCTCCGTGCCGCCAAGTTTTCAT

 60         70         80         90        100        110

TTTCCACCTTCTCTGCCTCCAGTCCCCCAGCCCCTGGCCGAGAGAAGGGTCTTACCG

    120        130        140        150        160        170

GCCGGGATTGCTGGAAACACCAAGAGGTGGTTTTTGTTTTTTAAAACTTCTGTTTCT

        180        190        200        210        220

TGGGAGGGGGTGTGGCGGGGCAGGATGAGCAACTCCGTTCCTCTGCTCTGTTTCTGG
                              --M--S--N--S--V--P--L--L--C--F--W--
230        240        250        260        270        280

AGCCTCTGCTATTGCTTTGCTGCGGGGAGCCCCGTACCTTTTGGTCCAGAGGGACGG
S--L--C--Y--C--F--A--A--G--S--P--V--P--F--G--P--E--G--R--
  290        300        310        320        330        340

CTGGAAGATAAGCTCCACAAACCCAAAGCTACACAGACTGAGGTCAAACCATCTGTG
L--E--D--K--L--H--K--P--K--A--T--Q--T--E--V--K--P--S--V--
      350        360        370        380        390

AGGTTTAACCTCCGCACCTCCAAGGACCCAGAGCATGAAGGATGCTACCTCTCCGTC
R--F--N--L--R--T--S--K--D--P--E--H--E--G--C--Y--L--S--V--
400        410        420        430        440        450

GGCCACAGCCAGCCCTTAGAAGACTGCAGTTTCAACATGACAGCTAAAACCTTTTTC
G--H--S--Q--P--L--E--D--C--S--F--N--M--T--A--K--T--F--F--
 460        470        480        490        500        510

ATCATTCACGGATGGACGATGAGCGGTATCTTTGAAAACTGGCTGCACAAACTCGTG
I--I--H--G--W--T--M--S--G--I--F--E--N--W--L--H--K--L--V--
    520        530        540        550        560        570

TCAGCCCTGCACACAAGAGAGAAAGACGCCAATGTAGTTGTGGTTGACTGGCTCCCC
S--A--L--H--T--R--E--K--D--A--N--V--V--V--V--D--W--L--P--
        580        590        600        610        620

CTGGCCCACCAGCTTTACACGGATGCGGTCAATAATACCAGGGTGGTGGGACACAGC
L--A--H--Q--L--Y--T--D--A--V--N--N--T--R--V--V--G--H--S--
630        640        650        660        670        680

ATTGCCAGGATGCTCGACTGGCTGCAGGAGAAGGACGATTTTTCTCTCGGGAATGTC
I--A--R--M--L--D--W--L--Q--E--K--D--D--F--S--L--G--N--V--
   690        700        710        720        730        740
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FIG. 1A

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CACTTGATCGGCTACAGCCTCGGAGCGCACGTGGCCGGGTATGCAGGCAACTTCGTG
H--L--I--G--Y--S--L--G--A--H--V--A--G--Y--A--G--N--F--V--
      750       760       770       780       780

AAAGGAACGGTGGGCCGAATCACAGGTTTGGATCCTGCCGGGCCCATGTTTGAAGGG
K--G--T--V--G--R--I--T--G--L--D--P--A--G--P--M--F--E--G--
800       810       820       830       840       850

GCCGACATCCACAAGAGGCTCTCTCCGGACGATGCAGATTTTGTGGATGTCCTCCAC
A--D--I--H--K--R--L--S--P--D--D--A--D--F--V--D--V--L--H--
  860       870       880       890       900       910

ACCTACACGCGTTCCTTCGGCTTGAGCATTGGTATTCAGATGCCTGTGGGCCACATT
T--Y--T--R--S--F--G--L--S--I--G--I--Q--M--P--V--G--H--I--
      920       930       940       950       960

GACATCTACCCCAATGGGGGTGACTTCCAGCCAGGCTGTGGACTCAACGATGTCTTG
D--I--Y--P--N--G--G--D--F--Q--P--G--C--G--L--N--D--V--L--
970       980       990       1000      1010      1020

GGATCAATTGCATATGGAACAATCACAGAGGTGGTAAAATGTGAGCATGAGCGAGCC
G--S--I--A--Y--G--T--I--T--E--V--V--K--C--E--H--E--R--A--
1030      1040      1050      1060      1070      1080

GTCCACCTCTTTGTTGACTCTCTGGTGAATCAGGACAAGCCGAGTTTTGCCTTCCAG
V--H--L--F--V--D--S--L--V--N--Q--D--K--P--S--F--A--F--Q--
   1090      1100      1110      1120      1130      1140

TGCACTGACTCCAATCGCTTCAAAAAGGGGATCTGTCTGAGCTGCCGCAAGAACCGT
C--T--D--S--N--R--F--K--K--G--I--C--L--S--C--R--K--N--R--
      1150      1160      1170      1180      1190

TGTAATAGCATTGGCTACAATGCCAAGAAAATGAGGAACAAGAGGAACAGCAAAATG
C--N--S--I--G--Y--N--A--K--K--M--R--N--K--R--N--S--K--M--
1200      1210      1220      1230      1240      1250

TACCTAAAAACCCGGGCAGGCATGCCTTTCAGAGTTTACCATTATCAGATGAAAATC
Y--L--K--T--R--A--G--M--P--F--R--V--Y--H--Y--Q--M--K--I--
  1260      1270      1280      1290      1300      1310

CATGTCTTCAGTTACAAGAACATGGGAGAAATTGAGCCCACCTTTTACGTCACCCTT
H--V--F--S--Y--K--N--M--G--E--I--E--P--T--F--Y--V--T--L--
      1320      1330      1340      1350      1360

TATGGCACTAATGCAGATTCCCAGACTCTGCCACTGGAAATAGTGGAGCGGATCGAG
Y--G--T--N--A--D--S--Q--T--L--P--L--E--I--V--E--R--I--E--
1370      1380      1390      1400      1410      1420

CAGAATGCCACCAACACCTTCCTGGTCTACACCGAGGAGGACTTGGGAGACCTCTTG
Q--N--A--T--N--T--F--L--V--Y--T--E--E--D--L--G--D--L--L--
  1430      1440      1450      1460      1470      1480
```

FIG. 1B

```
AAGATCCAGCTCACCTGGGAGGGGGCCTCTCAGTCTTGGTACAACCTGTGGAAGGAG
K--I--Q--L--T--W--E--G--A--S--Q--S--W--Y--N--L--W--K--E--
     1490       1500       1510       1520       1530

TTTCGCAGCTACCTGTCTCAACCCCGCAACCCCGGACGGGAGCTGAATATCAGGCGC
F--R--S--Y--L--S--Q--P--R--N--P--G--R--E--L--N--I--R--R--
1540       1550       1560       1570       1580       1590

ATCCGGGTGAAGTCTGGGGAAACCCAGCGGAAACTGACATTTTGTACAGAAGACCCT
I--R--V--K--S--G--E--T--Q--R--K--L--T--F--C--T--E--D--P--
1600       1610       1620       1630       1640       1650

GAGAACACCAGCATATCCCCAGGCCGGGAGCTCTGGTTTCGCAAGTGTCGGGATGGC
E--N--T--S--I--S--P--G--R--E--L--W--F--R--K--C--R--D--G--
   1660       1670       1680       1690       1700       1710

TGGAGGATGAAAAACGAAACCAGTCCCACTGTGGAGCTTCCCTGA
W--R--M--K--N--E--T--S--P--T--V--E--L--P--*--
       1720       1730       1740       1750
```

FIG.1C

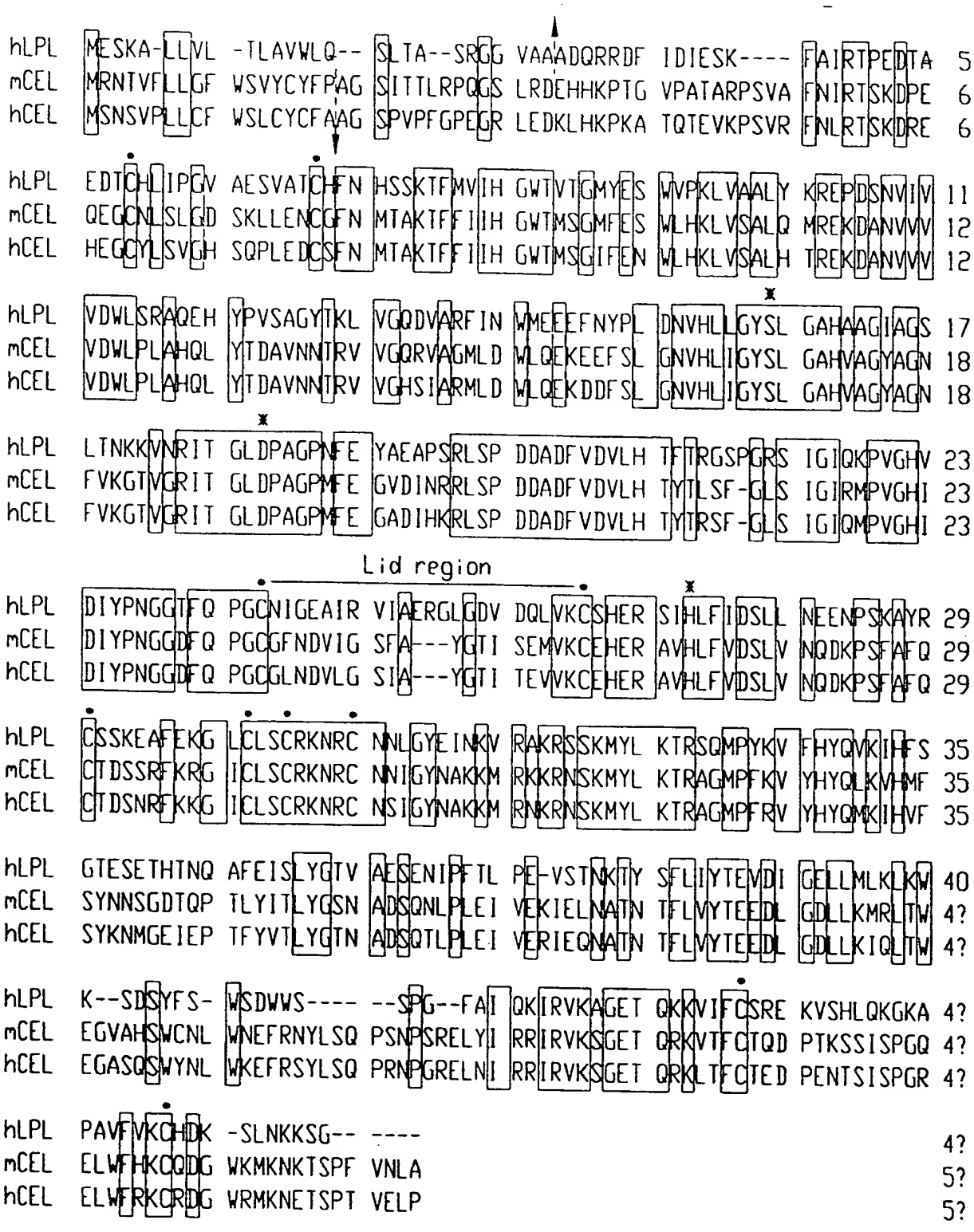

FIG.2

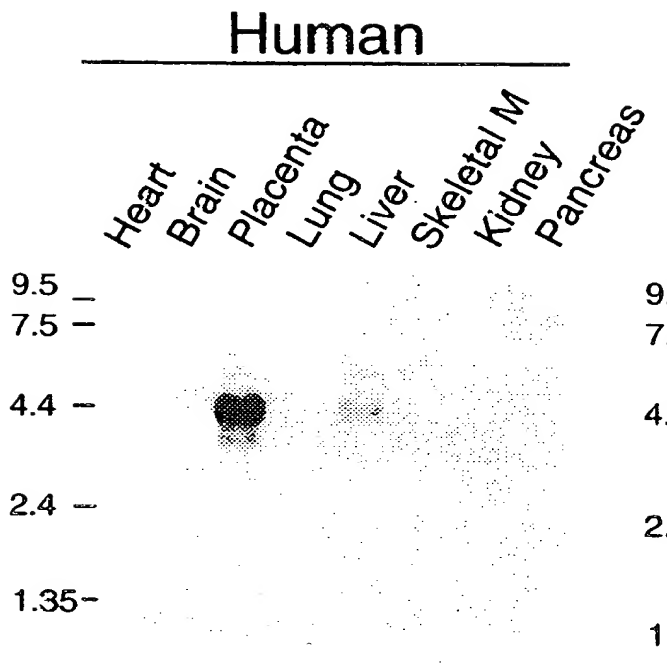

FIG.3A

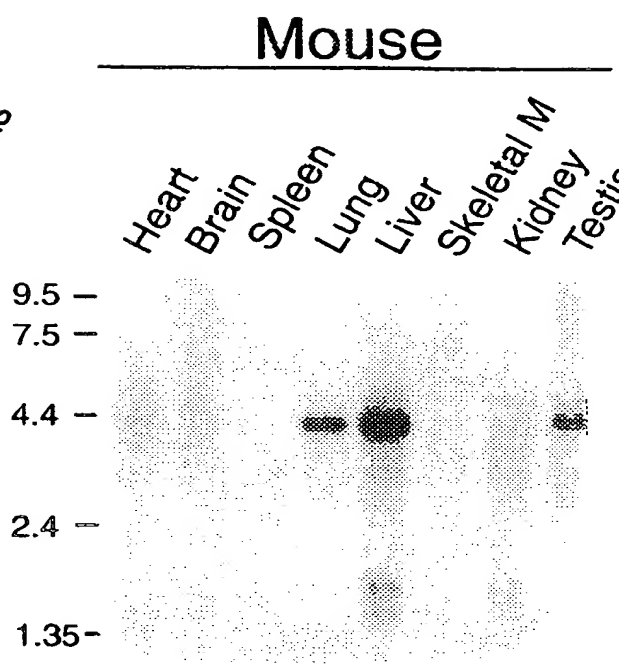

FIG.3B

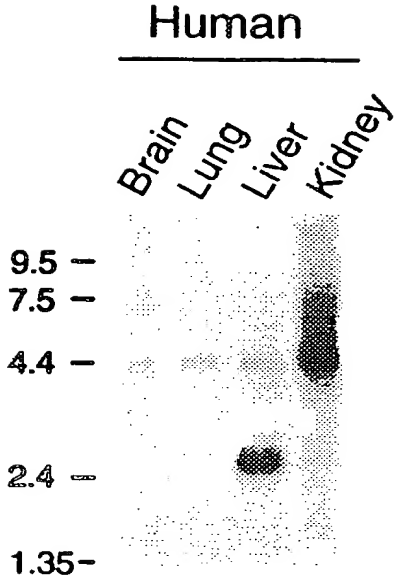
Human
Brain
Lung
Liver
Kidney
9.5
7.5
4.4
2.4
1.35

FIG.4A

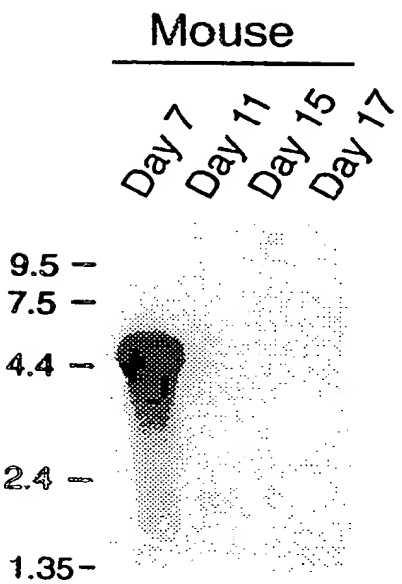
Mouse
Day 7
Day 11
Day 15
Day 17
9.5
7.5
4.4
2.4
1.35

FIG.4B

SEQUENCE LISTING

<110> Hirata, Ken-ichi
Quertermous, Thomas
Cioffi, Joseph
Zupancic, Thomas

<120> A LIPOPROTEIN LIPASE EXPRESSED IN ENDOTHELIAL CELLS AND METHODS FOR ITS USE

<130> 8907-0079-999

<160> 10

<170> FastSEQ for Windows Version 3.0

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<213> Homo Sapien

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<222> (253)...(1752)

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ccgttgacac tcgctccctg ccaccgcccg ggctccgtgc cgccaagttt tcatttttcca     120
ccttctctgc ctccagtccc ccagcccctg gccgagagaa gggtcttacc ggccgggatt      180
gctggaaaca ccaagaggtg gtttttgttt tttaaaactt ctgtttcttg ggaggggggtg     240
tggcggggca gg atg agc aac tcc gtt cct ctg ctc tgt ttc tgg agc ctc      291
              Met Ser Asn Ser Val Pro Leu Leu Cys Phe Trp Ser Leu
               1               5                  10

tgc tat tgc ttt gct gcg ggg agc ccc gta cct ttt ggt cca gag gga        339
Cys Tyr Cys Phe Ala Ala Gly Ser Pro Val Pro Phe Gly Pro Glu Gly
     15                  20                  25

cgg ctg gaa gat aag ctc cac aaa ccc aaa gct aca cag act gag gtc        387
Arg Leu Glu Asp Lys Leu His Lys Pro Lys Ala Thr Gln Thr Glu Val
 30                  35                  40                  45

aaa cca tct gtg agg ttt aac ctc cgc acc tcc aag gac cca gag cat        435
Lys Pro Ser Val Arg Phe Asn Leu Arg Thr Ser Lys Asp Pro Glu His
                50                  55                  60

gaa gga tgc tac ctc tcc gtc ggc cac agc cag ccc tta gaa gac tgc        483
Glu Gly Cys Tyr Leu Ser Val Gly His Ser Gln Pro Leu Glu Asp Cys
            65                  70                  75

agt ttc aac atg aca gct aaa acc ttt ttc atc att cac gga tgg acg        531
Ser Phe Asn Met Thr Ala Lys Thr Phe Phe Ile Ile His Gly Trp Thr
        80                  85                  90
```

```
atg agc ggt atc ttt gaa aac tgg ctg cac aaa ctc gtg tca gcc ctg      579
Met Ser Gly Ile Phe Glu Asn Trp Leu His Lys Leu Val Ser Ala Leu
 95                  100                 105

cac aca aga gag aaa gac gcc aat gta gtt gtg gtt gac tgg ctc ccc      627
His Thr Arg Glu Lys Asp Ala Asn Val Val Val Val Asp Trp Leu Pro
110                 115                 120                 125

ctg gcc cac cag ctt tac acg gat gcg gtc aat aat acc agg gtg gtg      675
Leu Ala His Gln Leu Tyr Thr Asp Ala Val Asn Asn Thr Arg Val Val
            130                 135                 140

gga cac agc att gcc agg atg ctc gac tgg ctg cag gag aag gac gat      723
Gly His Ser Ile Ala Arg Met Leu Asp Trp Leu Gln Glu Lys Asp Asp
        145                 150                 155

ttt tct ctc ggg aat gtc cac ttg atc ggc tac agc ctc gga gcg cac      771
Phe Ser Leu Gly Asn Val His Leu Ile Gly Tyr Ser Leu Gly Ala His
    160                 165                 170

gtg gcc ggg tat gca ggc aac ttc gtg aaa gga acg gtg ggc cga atc      819
Val Ala Gly Tyr Ala Gly Asn Phe Val Lys Gly Thr Val Gly Arg Ile
175                 180                 185

aca ggt ttg gat cct gcc ggg ccc atg ttt gaa ggg gcc gac atc cac      867
Thr Gly Leu Asp Pro Ala Gly Pro Met Phe Glu Gly Ala Asp Ile His
190                 195                 200                 205

aag agg ctc tct ccg gac gat gca gat ttt gtg gat gtc ctc cac acc      915
Lys Arg Leu Ser Pro Asp Asp Ala Asp Phe Val Asp Val Leu His Thr
            210                 215                 220

tac acg cgt tcc ttc ggc ttg agc att ggt att cag atg cct gtg ggc      963
Tyr Thr Arg Ser Phe Gly Leu Ser Ile Gly Ile Gln Met Pro Val Gly
        225                 230                 235

cac att gac atc tac ccc aat ggg ggt gac ttc cag cca ggc tgt gga     1011
His Ile Asp Ile Tyr Pro Asn Gly Gly Asp Phe Gln Pro Gly Cys Gly
    240                 245                 250

ctc aac gat gtc ttg gga tca att gca tat gga aca atc aca gag gtg     1059
Leu Asn Asp Val Leu Gly Ser Ile Ala Tyr Gly Thr Ile Thr Glu Val
255                 260                 265

gta aaa tgt gag cat gag cga gcc gtc cac ctc ttt gtt gac tct ctg     1107
Val Lys Cys Glu His Glu Arg Ala Val His Leu Phe Val Asp Ser Leu
270                 275                 280                 285

gtg aat cag gac aag ccg agt ttt gcc ttc cag tgc act gac tcc aat     1155
Val Asn Gln Asp Lys Pro Ser Phe Ala Phe Gln Cys Thr Asp Ser Asn
            290                 295                 300

cgc ttc aaa aag ggg atc tgt ctg agc tgc cgc aag aac cgt tgt aat     1203
Arg Phe Lys Lys Gly Ile Cys Leu Ser Cys Arg Lys Asn Arg Cys Asn
        305                 310                 315
```

```
agc att ggc tac aat gcc aag aaa atg agg aac aag agg aac agc aaa      1251
Ser Ile Gly Tyr Asn Ala Lys Lys Met Arg Asn Lys Arg Asn Ser Lys
        320                 325                 330

atg tac cta aaa acc cgg gca ggc atg cct ttc aga gtt tac cat tat      1299
Met Tyr Leu Lys Thr Arg Ala Gly Met Pro Phe Arg Val Tyr His Tyr
    335                 340                 345

cag atg aaa atc cat gtc ttc agt tac aag aac atg gga gaa att gag      1347
Gln Met Lys Ile His Val Phe Ser Tyr Lys Asn Met Gly Glu Ile Glu
350                 355                 360                 365

ccc acc ttt tac gtc acc ctt tat ggc act aat gca gat tcc cag act      1395
Pro Thr Phe Tyr Val Thr Leu Tyr Gly Thr Asn Ala Asp Ser Gln Thr
                370                 375                 380

ctg cca ctg gaa ata gtg gag cgg atc gag cag aat gcc acc aac acc      1443
Leu Pro Leu Glu Ile Val Glu Arg Ile Glu Gln Asn Ala Thr Asn Thr
            385                 390                 395

ttc ctg gtc tac acc gag gag gac ttg gga gac ctc ttg aag atc cag      1491
Phe Leu Val Tyr Thr Glu Glu Asp Leu Gly Asp Leu Leu Lys Ile Gln
        400                 405                 410

ctc acc tgg gag ggg gcc tct cag tct tgg tac aac ctg tgg aag gag      1539
Leu Thr Trp Glu Gly Ala Ser Gln Ser Trp Tyr Asn Leu Trp Lys Glu
    415                 420                 425

ttt cgc agc tac ctg tct caa ccc cgc aac ccc gga cgg gag ctg aat      1587
Phe Arg Ser Tyr Leu Ser Gln Pro Arg Asn Pro Gly Arg Glu Leu Asn
430                 435                 440                 445

atc agg cgc atc cgg gtg aag tct ggg gaa acc cag cgg aaa ctg aca      1635
Ile Arg Arg Ile Arg Val Lys Ser Gly Glu Thr Gln Arg Lys Leu Thr
                450                 455                 460

ttt tgt aca gaa gac cct gag aac acc agc ata tcc cca ggc cgg gag      1683
Phe Cys Thr Glu Asp Pro Glu Asn Thr Ser Ile Ser Pro Gly Arg Glu
            465                 470                 475

ctc tgg ttt cgc aag tgt cgg gat ggc tgg agg atg aaa aac gaa acc      1731
Leu Trp Phe Arg Lys Cys Arg Asp Gly Trp Arg Met Lys Asn Glu Thr
        480                 485                 490

agt ccc act gtg gag ctt ccc tga                                     1755
Ser Pro Thr Val Glu Leu Pro
    495                 500
```

<210> 2
<211> 500
<212> PRT
<213> Homo Sapien

<400> 2

```
Met Ser Asn Ser Val Pro Leu Leu Cys Phe Trp Ser Leu Cys Tyr Cys
```

```
 1               5                  10                  15
Phe Ala Ala Gly Ser Pro Val Pro Phe Gly Pro Glu Gly Arg Leu Glu
            20                  25                  30
Asp Lys Leu His Lys Pro Lys Ala Thr Gln Thr Glu Val Lys Pro Ser
        35                  40                  45
Val Arg Phe Asn Leu Arg Thr Ser Lys Asp Pro Glu His Glu Gly Cys
    50                  55                  60
Tyr Leu Ser Val Gly His Ser Gln Pro Leu Glu Asp Cys Ser Phe Asn
65                  70                  75                  80
Met Thr Ala Lys Thr Phe Phe Ile Ile His Gly Trp Thr Met Ser Gly
                85                  90                  95
Ile Phe Glu Asn Trp Leu His Lys Leu Val Ser Ala Leu His Thr Arg
            100                 105                 110
Glu Lys Asp Ala Asn Val Val Val Val Asp Trp Leu Pro Leu Ala His
        115                 120                 125
Gln Leu Tyr Thr Asp Ala Val Asn Asn Thr Arg Val Val Gly His Ser
    130                 135                 140
Ile Ala Arg Met Leu Asp Trp Leu Gln Glu Lys Asp Asp Phe Ser Leu
145                 150                 155                 160
Gly Asn Val His Leu Ile Gly Tyr Ser Leu Gly Ala His Val Ala Gly
                165                 170                 175
Tyr Ala Gly Asn Phe Val Lys Gly Thr Val Gly Arg Ile Thr Gly Leu
            180                 185                 190
Asp Pro Ala Gly Pro Met Phe Glu Gly Ala Asp Ile His Lys Arg Leu
        195                 200                 205
Ser Pro Asp Asp Ala Asp Phe Val Asp Val Leu His Thr Tyr Thr Arg
    210                 215                 220
Ser Phe Gly Leu Ser Ile Gly Ile Gln Met Pro Val Gly His Ile Asp
225                 230                 235                 240
Ile Tyr Pro Asn Gly Gly Asp Phe Gln Pro Gly Cys Gly Leu Asn Asp
                245                 250                 255
Val Leu Gly Ser Ile Ala Tyr Gly Thr Ile Thr Glu Val Val Lys Cys
            260                 265                 270
Glu His Glu Arg Ala Val His Leu Phe Val Asp Ser Leu Val Asn Gln
        275                 280                 285
Asp Lys Pro Ser Phe Ala Phe Gln Cys Thr Asp Ser Asn Arg Phe Lys
    290                 295                 300
Lys Gly Ile Cys Leu Ser Cys Arg Lys Asn Arg Cys Asn Ser Ile Gly
305                 310                 315                 320
Tyr Asn Ala Lys Lys Met Arg Asn Lys Arg Asn Ser Lys Met Tyr Leu
                325                 330                 335
Lys Thr Arg Ala Gly Met Pro Phe Arg Val Tyr His Tyr Gln Met Lys
            340                 345                 350
Ile His Val Phe Ser Tyr Lys Asn Met Gly Glu Ile Glu Pro Thr Phe
        355                 360                 365
Tyr Val Thr Leu Tyr Gly Thr Asn Ala Asp Ser Gln Thr Leu Pro Leu
    370                 375                 380
Glu Ile Val Glu Arg Ile Glu Gln Asn Ala Thr Asn Thr Phe Leu Val
385                 390                 395                 400
Tyr Thr Glu Glu Asp Leu Gly Asp Leu Leu Lys Ile Gln Leu Thr Trp
                405                 410                 415
Glu Gly Ala Ser Gln Ser Trp Tyr Asn Leu Trp Lys Glu Phe Arg Ser
            420                 425                 430
Tyr Leu Ser Gln Pro Arg Asn Pro Gly Arg Glu Leu Asn Ile Arg Arg
        435                 440                 445
Ile Arg Val Lys Ser Gly Glu Thr Gln Arg Lys Leu Thr Phe Cys Thr
```

```
    450                 455                 460
Glu Asp Pro Glu Asn Thr Ser Ile Ser Pro Gly Arg Glu Leu Trp Phe
465                 470                 475                 480
Arg Lys Cys Arg Asp Gly Trp Arg Met Lys Asn Glu Thr Ser Pro Thr
                485                 490                 495
Val Glu Leu Pro
            500
```

<210> 3
<211> 500
<212> PRT
<213> Mus musculus

<400> 3

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Met Arg Asn Thr Val Phe Leu Leu Gly Phe Trp Ser Val Tyr Cys Tyr
 1               5                  10                  15
Phe Pro Ala Gly Ser Ile Thr Thr Leu Arg Pro Gln Gly Ser Leu Arg
            20                  25                  30
Asp Glu His His Lys Pro Thr Gly Val Pro Ala Thr Ala Arg Pro Ser
        35                  40                  45
Val Ala Phe Asn Ile Arg Thr Ser Lys Asp Pro Glu Gln Glu Gly Cys
    50                  55                  60
Asn Leu Ser Leu Gly Asp Ser Lys Leu Leu Glu Asn Cys Gly Phe Asn
65                  70                  75                  80
Met Thr Ala Lys Thr Phe Phe Ile Ile His Gly Trp Thr Met Ser Gly
                85                  90                  95
Met Phe Glu Ser Trp Leu His Lys Leu Val Ser Ala Leu Gln Met Arg
            100                 105                 110
Glu Lys Asp Ala Asn Val Val Val Val Asp Trp Leu Pro Leu Ala His
        115                 120                 125
Gln Leu Tyr Thr Asp Ala Val Asn Asn Thr Arg Val Val Gly Gln Arg
    130                 135                 140
Val Ala Gly Met Leu Asp Trp Leu Gln Glu Lys Glu Glu Phe Ser Leu
145                 150                 155                 160
Gly Asn Val His Leu Ile Gly Tyr Ser Leu Gly Ala His Val Ala Gly
                165                 170                 175
Tyr Ala Gly Asn Phe Val Lys Gly Thr Val Gly Arg Ile Thr Gly Leu
            180                 185                 190
Asp Pro Ala Gly Pro Met Phe Glu Gly Val Asp Ile Asn Arg Arg Leu
        195                 200                 205
Ser Pro Asp Asp Ala Asp Phe Val Asp Val Leu His Thr Tyr Thr Leu
    210                 215                 220
Ser Phe Gly Leu Ser Ile Gly Ile Arg Met Pro Val Gly His Ile Asp
225                 230                 235                 240
Ile Tyr Pro Asn Gly Gly Asp Phe Gln Pro Gly Cys Gly Phe Asn Asp
                245                 250                 255
Val Ile Gly Ser Phe Ala Tyr Gly Thr Ile Ser Glu Met Val Lys Cys
            260                 265                 270
Glu His Glu Arg Ala Val His Leu Phe Val Asp Ser Leu Val Asn Gln
        275                 280                 285
Asp Lys Pro Ser Phe Ala Phe Gln Cys Thr Asp Ser Ser Arg Phe Lys
    290                 295                 300
Arg Gly Ile Cys Leu Ser Cys Arg Lys Asn Arg Cys Asn Asn Ile Gly
305                 310                 315                 320
Tyr Asn Ala Lys Lys Met Arg Lys Lys Arg Asn Ser Lys Met Tyr Leu
                325                 330                 335
```

```
Lys Thr Arg Ala Gly Met Pro Phe Lys Val Tyr His Tyr Gln Leu Lys
            340                 345                 350
Val His Met Phe Ser Tyr Asn Asn Ser Gly Asp Thr Gln Pro Thr Leu
        355                 360                 365
Tyr Ile Thr Leu Tyr Gly Ser Asn Ala Asp Ser Gln Asn Leu Pro Leu
    370                 375                 380
Glu Ile Val Glu Lys Ile Glu Leu Asn Ala Thr Asn Thr Phe Leu Val
385                 390                 395                 400
Tyr Thr Glu Glu Asp Leu Gly Asp Leu Leu Lys Met Arg Leu Thr Trp
                405                 410                 415
Glu Gly Val Ala His Ser Trp Cys Asn Leu Trp Asn Glu Phe Arg Asn
            420                 425                 430
Tyr Leu Ser Gln Pro Ser Asn Pro Ser Arg Glu Leu Tyr Ile Arg Arg
        435                 440                 445
Ile Arg Val Lys Ser Gly Glu Thr Gln Arg Lys Val Thr Phe Cys Thr
    450                 455                 460
Gln Asp Pro Thr Lys Ser Ser Ile Ser Pro Gly Gln Glu Leu Trp Phe
465                 470                 475                 480
His Lys Cys Gln Asp Gly Trp Lys Met Lys Asn Lys Thr Ser Pro Phe
                485                 490                 495
Val Asn Leu Ala
            500
```

<210> 4
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<212> PRT
<213> Homo sapien

<400> 4

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 1                5                  10                  15
Phe Ala Ala Gly Ser Pro Val Pro Phe Gly Pro Glu Gly Arg Leu Glu
            20                  25                  30
Asp Lys Leu His Lys Pro Lys Ala Thr Gln Thr Glu Val Lys Pro Ser
        35                  40                  45
Val Arg Phe Asn Leu Arg Thr Ser Lys Asp Pro Glu His Glu Gly Cys
    50                  55                  60
Tyr Leu Ser Val Gly His Ser Gln Pro Leu Glu Asp Cys Ser Phe Asn
65                  70                  75                  80
Met Thr Ala Lys Thr Phe Phe Ile Ile His Gly Trp Thr Met Ser Gly
                85                  90                  95
Ile Phe Glu Asn Trp Leu His Lys Leu Val Ser Ala Leu His Thr Arg
            100                 105                 110
Glu Lys Asp Ala Asn Val Val Val Val Asp Trp Leu Pro Leu Ala His
        115                 120                 125
Gln Leu Tyr Thr Asp Ala Val Asn Asn Thr Arg Val Val Gly His Ser
    130                 135                 140
Ile Ala Arg Met Leu Asp Trp Leu Gln Glu Lys Asp Asp Phe Ser Leu
145                 150                 155                 160
Gly Asn Val His Leu Ile Gly Tyr Ser Leu Gly Ala His Val Ala Gly
                165                 170                 175
Tyr Ala Gly Asn Phe Val Lys Gly Thr Val Gly Arg Ile Thr Gly Leu
            180                 185                 190
Asp Pro Ala Gly Pro Met Phe Glu Gly Ala Asp Ile His Lys Arg Leu
        195                 200                 205
Ser Pro Asp Asp Ala Asp Phe Val Asp Val Leu His Thr Tyr Thr Arg
```

```
    210                 215                 220
Ser Phe Gly Leu Ser Ile Gly Ile Gln Met Pro Val Gly His Ile Asp
225                 230                 235                 240
Ile Tyr Pro Asn Gly Gly Asp Phe Gln Pro Gly Cys Gly Leu Asn Asp
                245                 250                 255
Val Leu Gly Ser Ile Ala Tyr Gly Thr Ile Thr Glu Val Val Lys Cys
            260                 265                 270
Glu His Glu Arg Ala Val His Leu Phe Val Asp Ser Leu Val Asn Gln
        275                 280                 285
Asp Lys Pro Ser Phe Ala Phe Gln Cys Thr Asp Ser Asn Arg Phe Lys
    290                 295                 300
Lys Gly Ile Cys Leu Ser Cys Arg Lys Asn Arg Cys Asn Ser Ile Gly
305                 310                 315                 320
Tyr Asn Ala Lys Lys Met Arg Asn Lys Arg Asn Ser Lys Met Tyr Leu
                325                 330                 335
Lys Thr Arg Ala Gly Met Pro Phe Arg Val Tyr His Tyr Gln Met Lys
            340                 345                 350
Ile His Val Phe Ser Tyr Lys Asn Met Gly Glu Ile Glu Pro Thr Phe
        355                 360                 365
Tyr Val Thr Leu Tyr Gly Thr Asn Ala Asp Ser Gln Thr Leu Pro Leu
    370                 375                 380
Glu Ile Val Glu Arg Ile Glu Gln Asn Ala Thr Asn Thr Phe Leu Val
385                 390                 395                 400
Tyr Thr Glu Glu Asp Leu Gly Asp Leu Leu Lys Ile Gln Leu Thr Trp
                405                 410                 415
Glu Gly Ala Ser Gln Ser Trp Tyr Asn Leu Trp Lys Glu Phe Arg Ser
            420                 425                 430
Tyr Leu Ser Gln Pro Arg Asn Pro Gly Arg Glu Leu Asn Ile Arg Arg
        435                 440                 445
Ile Arg Val Lys Ser Gly Glu Thr Gln Arg Lys Leu Thr Phe Cys Thr
    450                 455                 460
Glu Asp Pro Glu Asn Thr Ser Ile Ser Pro Gly Arg Glu Leu Trp Phe
465                 470                 475                 480
Arg Lys Cys Arg Asp Gly Trp Arg Met Lys Asn Glu Thr Ser Pro Thr
                485                 490                 495
Val Glu Leu Pro
            500
```

<210> 5
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<213> Mus musculus

<220>
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<400> 5

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tccggcccga cttcgcagcg gctctgggaa aggacaggtc gggctgggca gcgtgtccct     120
ccacacgctc gctttcccgg tcaccgctcg cagcgctacc aaccttccag gttctgccag     180
ctgtccaccc catcaccttc tggccgacac ccggcctgtc cacttctagt ctctggaggt     240
tttagtggtt tcaaaccaaa ccaacccaaa ccaacccaac aacaaaaaag cccaaaccaa     300
aaacctgctt gagaggaggg ggcgtggcgg ggaagg atg cga aac acg gtt ttc       354
                                        Met Arg Asn Thr Val Phe
                                         1               5
```

```
ctg ctc ggc ttt tgg agc gtc tat tgt tac ttc ccg gcg gga agt atc      402
Leu Leu Gly Phe Trp Ser Val Tyr Cys Tyr Phe Pro Ala Gly Ser Ile
            10                  15                  20

aca acc ttg cgt ccc cag ggg tcg ctg cga gat gag cat cat aaa ccc      450
Thr Thr Leu Arg Pro Gln Gly Ser Leu Arg Asp Glu His His Lys Pro
        25                  30                  35

act gga gta cca gct acc gcc aga ccc tct gtg gct ttt aac atc cgc      498
Thr Gly Val Pro Ala Thr Ala Arg Pro Ser Val Ala Phe Asn Ile Arg
    40                  45                  50

act tct aag gac cca gag cag gaa ggg tgt aat ctc tcc ctt ggt gac      546
Thr Ser Lys Asp Pro Glu Gln Glu Gly Cys Asn Leu Ser Leu Gly Asp
55                  60                  65                  70

agc aaa ctc tta gaa aac tgt ggc ttc aac atg aca gcc aaa acc ttc      594
Ser Lys Leu Leu Glu Asn Cys Gly Phe Asn Met Thr Ala Lys Thr Phe
                75                  80                  85

ttc atc att cat gga tgg acg atg agt ggc atg ttt gag agc tgg ctg      642
Phe Ile Ile His Gly Trp Thr Met Ser Gly Met Phe Glu Ser Trp Leu
            90                  95                  100

cat aaa ctt gta tca gcc ctg cag atg aga gag aaa gat gct aac gtc      690
His Lys Leu Val Ser Ala Leu Gln Met Arg Glu Lys Asp Ala Asn Val
        105                 110                 115

gtg gtg gtt gac tgg ctg ccc ctg gct cat cag ctg tac acg gat gca      738
Val Val Val Asp Trp Leu Pro Leu Ala His Gln Leu Tyr Thr Asp Ala
    120                 125                 130

gtc aat aac acc agg gtg gtg gga cag aga gta gct ggg atg ctt gac      786
Val Asn Asn Thr Arg Val Val Gly Gln Arg Val Ala Gly Met Leu Asp
135                 140                 145                 150

tgg ctg cag gag aag gaa gag ttc tct ctt ggg aac gtt cac ttg att      834
Trp Leu Gln Glu Lys Glu Glu Phe Ser Leu Gly Asn Val His Leu Ile
                155                 160                 165

ggc tac agc ctt gga gca cac gtg gct gga tac gct ggc aac ttt gtg      882
Gly Tyr Ser Leu Gly Ala His Val Ala Gly Tyr Ala Gly Asn Phe Val
            170                 175                 180

aaa gga aca gtg ggc agg atc act ggt ctg gat ccc gcg ggt ccc atg      930
Lys Gly Thr Val Gly Arg Ile Thr Gly Leu Asp Pro Ala Gly Pro Met
        185                 190                 195

ttt gaa ggg gtg gac atc aac aga agg ctg tcc ccg gac gat gca gac      978
Phe Glu Gly Val Asp Ile Asn Arg Arg Leu Ser Pro Asp Asp Ala Asp
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ttt gtg gat gtc ctg cat acc tac acg ctg tcc ttt ggc ttg agc att     1026
Phe Val Asp Val Leu His Thr Tyr Thr Leu Ser Phe Gly Leu Ser Ile
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ggg att cgg atg cct gtg ggt cac att gac atc tat ccc aat ggc ggt      1074
Gly Ile Arg Met Pro Val Gly His Ile Asp Ile Tyr Pro Asn Gly Gly
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gac ttc cag cca ggc tgt gga ttc aat gat gtc atc gga tct ttt gca      1122
Asp Phe Gln Pro Gly Cys Gly Phe Asn Asp Val Ile Gly Ser Phe Ala
            250                 255                 260

tat gga aca atc tca gag atg gtg aaa tgc gag cac gag cga gcc gta      1170
Tyr Gly Thr Ile Ser Glu Met Val Lys Cys Glu His Glu Arg Ala Val
        265                 270                 275

cac ctc ttt gtc gac tct ctg gtg aat cag gac aag ccc agc ttt gcc      1218
His Leu Phe Val Asp Ser Leu Val Asn Gln Asp Lys Pro Ser Phe Ala
    280                 285                 290

ttc cag tgc aca gac tcc agc cgc ttc aaa agg gga atc tgc ctc agc      1266
Phe Gln Cys Thr Asp Ser Ser Arg Phe Lys Arg Gly Ile Cys Leu Ser
295                 300                 305                 310

tgc cgg aag aac cgt tgt aat aac att ggc tac aac gcc aag aaa atg      1314
Cys Arg Lys Asn Arg Cys Asn Asn Ile Gly Tyr Asn Ala Lys Lys Met
                315                 320                 325

aga aag aag agg aat agc aaa atg tat tta aaa acc cgg gct ggc atg      1362
Arg Lys Lys Arg Asn Ser Lys Met Tyr Leu Lys Thr Arg Ala Gly Met
            330                 335                 340

cct ttc aaa gtt tac cat tac cag ctg aaa gtt cac atg ttc tct tac      1410
Pro Phe Lys Val Tyr His Tyr Gln Leu Lys Val His Met Phe Ser Tyr
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aat aac agt ggg gac acc cag ccc acc ctc tac att acc ctg tat ggt      1458
Asn Asn Ser Gly Asp Thr Gln Pro Thr Leu Tyr Ile Thr Leu Tyr Gly
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agc aac gca gac tcc cag aac ctg ccc ttg gaa ata gtg gag aag att      1506
Ser Asn Ala Asp Ser Gln Asn Leu Pro Leu Glu Ile Val Glu Lys Ile
375                 380                 385                 390

gag ctg aat gcc aca aac acc ttc ctt gtc tac act gag gag gac ttg      1554
Glu Leu Asn Ala Thr Asn Thr Phe Leu Val Tyr Thr Glu Glu Asp Leu
                395                 400                 405

ggc gat ctc ttg aag atg cga ctt acc tgg gag ggg gta gcc cat tcc      1602
Gly Asp Leu Leu Lys Met Arg Leu Thr Trp Glu Gly Val Ala His Ser
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Trp Cys Asn Leu Trp Asn Glu Phe Arg Asn Tyr Leu Ser Gln Pro Ser
        425                 430                 435

aac ccc tcg agg gag ctg tac atc cgg cga att cgt gtc aaa tct ggg      1698
Asn Pro Ser Arg Glu Leu Tyr Ile Arg Arg Ile Arg Val Lys Ser Gly
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agc atc tcc cct ggc cag gag ctg tgg ttt cac aag tgt cag gat ggc      1794
Ser Ile Ser Pro Gly Gln Glu Leu Trp Phe His Lys Cys Gln Asp Gly
                475                 480                 485

tgg aaa atg aaa aac aaa acc agt ccc ttt gtg aac ttg gcc               1836
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tga                                                                  1839
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ct 62

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/27335

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/20, 1/20, 15/00; C07H 21/04; C07K 1/00; A61K 38/46

US CL :435/198, 252.3, 320.1; 536/23.2; 530/350; 424/94.6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/198, 252.3, 320.1; 536/23.2; 530/350; 424/94.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SIVARAM et al. Endothelial Cells Synthesize and Process Apolipoprotein B. Journal of Biological Chemistry 21 June 1996, Vol. 271, No. 25, pages 15261-15266, see the entire article.	1-4, 9-18 & 20-23
A	BUSCA et al. The Mutant Asn-291 fwdarw Ser Human Lipoprotein Lipase is Associated with Reduced Catalytic Activity and does not Influence Binding to Heparin. FEBS Letters 1995, Vol. 367, No. 3, pages 257-262, see the entire article.	1-4, 9-18 & 20-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 FEBRUARY 1999	Date of mailing of the international search report 02 MAR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer TEKCHAND SAIDHA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/27335

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 9-18 & 20-23

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/27335

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS and STN searches. STN files included - Medline, Caplus, Wpids, Biosis, Biotechds & Scisearch. Selected Search Terms included : Endothelial cell lipase? or EL, (human or murine) and (EL or endothelial cell? lipase?) and combinations of different invention related terms.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-4, 9-18 & 20-23, drawn to a polynucleotide encoding a human endothelial cell lipase (EL) of SE ID NO. 2, host cell, the protein and the method of making the protein.
Group II, claim(s) 5-8 & 19, drawn to polynucleotide (SEQ ID NO. 5) encoding murine endothelial cell lipase.
Group III, claim(s) 24-25, drawn to antiboby.
Group IV, claim(s)26, drawn to a method of diagnosing hypertriglyceridemia.
Group V, claim(s) 27, drawn to a method of diagnosing hypercholesterolemia.
Group VI, claim(s) 28, drawn to a method of treating hypertriglyceridemia.
Group VII, claim(s) 29, drawn to a method of treating hypercholesterolemia.
Group VIII, claim(s) 28, drawn to a method of treating cancer.
The inventions listed as Groups I-VIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The polynucleotide encoding the human EL, host cells, the polypeptide and the method of making the polypeptide of Group I and the polynucleotide encoding the murine EL of Group II are obtained from two different animal species and are materially different as revealed by their DNA and amino acid sequences.The polynucleotide of Group I & II and the antibodies of Group III do not require each other for their practice, have separate utilities, such as polynucleotides of Group I & II can be used for the recombinant production of protein versus the use of antibodies for protein detection, and are physically, biologically and chemically different from each other, and are subject to separate manufacture and sale from each other. Groups IV-VIII all employ the novel lipase or its nucleic acid, however, in view of 37 CFR 1.475 (b), when claims corresponding to different categories of invention are present then only (3) applies and additional methods of use are deemed to lack unity. Thus the various group discussed above show a lack of unity of invention.